The Lister Institute of Preventive Medicine.

Collected Papers,

No. 8.

Part II. Physiological, Zoological and Biochemical Papers.

LONDON:
—
1911-1912.

CONTENTS.

10. The Aetiology of the Deficiency Diseases. By CASIMIR FUNK. (*Journal of State Medicine*, Vol. 20, 1912).

11. The Constitution of Aminotyrosine and the Action of Oxydases on Some Tyrosine Derivatives. By CASIMIR FUNK. (*Transactions of the Chemical Society*, 1912).

12. Occurrence of Ferments in the Sterile Milk collected by Milking Tube from Cows and Goats. By A. HARDEN and JANET LANE-CLAYPON. (*Journal of Hygiene*, Vol. XII., 1912).

13. The Oxidation of Isolated Animal Tissues. By A. HARDEN and H. MACLEAN. (*Journal of Physiology*, Vol. XLIII., 1911).

14. The Bacterial Production of Acetylmethylcarbinol and 2·3-Butylene Glycol from various Substances. By A. HARDEN and DOROTHY NORRIS. (*Proceedings of Royal Society*, B. Vol. 84, 1912).

15. The Bacterial Production of Acetylmethylcarbinol and 2·3-Butylene Glycol from various Substances, Part II. By A. HARDEN and DOROTHY NORRIS. (*Proceedings of the Royal Society*, B. Vol. 85, 1912).

16. Action of Dissolved Substances upon the Autofermentation of Yeast. By A. HARDEN and S. G PAINE. (*Proceedings of Royal Society*, B. Vol. 84, 1912).

17. Der Mechanismus der alkoholischen Gärung. By A. HARDEN and W. J. YOUNG. (*Biochemischen Zeitschrift*, Bd. 40, 1912.)

18. Contributions to the Study of Pathogenic Amœbæ from Bombay. By W. GLEN LISTON and C. H. MARTIN. (*Quarterly Journal of Microscopical Science*, Vol. 57, 1911)

19. Further Observations on the Cæcal Parasites of Fowls, with some Reference to the Rectal Fauna of other Vertebrates. By C. H. MARTIN and MURIEL ROBERTSON. (*Quarterly Journal of Microscopical Science*, Vol. 57, 1911).

20. Speculations with regard to the Simplest Forms of Life and their origin on the Earth. (President's Address.) By E. A MINCHIN. (*Journal Quekett Microscopical Club*, 1912).

21. On the Occurrence of an Intracellular Stage in the Development of *Trypanosoma Lewisi* in the Rat-flea. By E. A. MINCHIN and J. D. THOMSON. (*British Medical Journal*, II., 1911).

22. Observations on the Trypanosome of the Little Owl (*Athene noctua*). By E. A. MINCHIN and H. M. WOODCOCK. (*Quarterly Journal of Microscopical Science*, Vol. 57, 1911).

23. On a Unique Pathological Condition in a Hare. By WILLIAM NICOLL. (*Proceedings of the Zoological Society of London*, 1911).

24. On Three new Trematodes from Reptiles. By WILLIAM NICOLL. (*Proceedings of the Zoological Society of London*, 1911).

25. **The Permeability of the Yeast-cell.** By S. G. PAINE. (*Proceedings of the Royal Society*, B. Vol. 84, 1911).

26. **On the Question of the Identity of Pepsin and Rennet.** By AGNES ELLEN PORTER. (*Journal of Physiology*, Vol. XLII., 1911).

27. **The Division of the Collar-cells of** *Calcarea heterocœla.* By MURIEL ROBERTSON. (*Quarterly Journal of Microscopical Science*, Vol. 57, 1911).

28. **The Development of a Leucocytozoon of Guinea-Pigs.** By E. H. ROSS. (*Annals of Tropical Medicine and Parasitology*, Vol. VI., No. I.B., May, 1912).

29. **An Iron-Haematein Stain, with Remarks on the Giemsa Stain.** By HARALD SEIDELIN. (*Parasitology*, Vol. IV., 1911).

30. **The Action of the Liver on the Simpler Sugars.** By IDA SMEDLEY. (*Journal of Physiology*, Vol. XLIV., 1912).

31. **The Chemical Action of** *Bacillus cloacæ* **(Jordan) on Glucose and Mannitol.** By J. THOMPSON. (*Proceedings of the Royal Society*, B. Vol. 84, 1912).

32. **A Reply to Miss Porter's note entitled "Some Remarks on the Genera** *Crithidia*, *Herpetomonas* **and** *Trypanosoma.*" By H. M. WOODCOCK (*Parasitology*, Vol. IV., 1911).

33. **On an unusual condition observed in** *Halteridium.* By H. M. WOODCOCK. (*Zoologischen Anzeiger*, Bd. XXXVIII, 1911).

Sonderabdruck aus d. Biochemischen Zeitschrift 40. Bd., S. 479.

Die vermeintliche Dioxyacetonbildung während der alkoholischen Gärung und die Wirkung von Tierkohle und von Methylphenylhydrazin auf Dioxyaceton.

Von

Frances Chick.

(Aus der Biochemischen Abteilung des Lister Instituts, London.)

(Eingegangen am 11. März 1912.)

P. Boysen-Jensen[1]) behauptet die Anwesenheit von Dioxyaceton in Kahlbaumscher Glucose nachgewiesen zu haben und ebenso die Bildung dieser Substanz, wenn Glucose durch Hefe vergoren wird. Die Gärung wurde in Gegenwart von Natriumsulfat oder Hydroxylaminchlorhydrat ausgeführt, um sie in dem Dioxyacetonstadium unterbrechen zu können. Man bestimmte das Dioxyaceton, indem man der verdächtigen, von Hefe abfiltrierten Flüssigkeit in 96%iger Essigsäure gelöstes Methylphenylhydrazin zufügte und sie in einem hermetisch verschlossenen Gefäß bei 20° einige Tage im Brutschrank stehen ließ. Es entstanden nach dieser Behandlung Krystalle, die abfiltriert ausgewaschen und getrocknet wurden. Nach Extraktion mit Alkohol wurde die alkoholische Lösung verdampft und der Rückstand als Methylphenylglycerosazon quantitativ bestimmt. Das erhaltene Produkt schmolz bei 127 bis 130° und zeigte die Zusammensetung der letztgenannten Substanz. Kahlbaumsche reine Glucose lieferte 0,22% dieses Stoffes. nach Behandlung mit Hefe 0,67%, der Unterschied ist also während der Gärung hinzugekommen. Da die Dioxyacetonbildung während des Gärprozesses eine bedeutsame Stütze für die Ansicht ist, daß wir in dieser Substanz ein Zwischenprodukt

[1]) P. Boysen-Jensen, Dissertation Kopenhagen 1910; Ber. d. Deutsch. bot. Ges. **26**, 666, 1908.

der alkoholischen Gärung von Glucose vor uns sehen, wurden die Versuche mit englischer, obergäriger Bierhefe wiederholt. Im Laufe dieser Untersuchungen stellte sich heraus, daß, wenn Dioxyaceton unter den von Jensen angegebenen Verdünnungs- und Temperaturbedingungen mit Methylphenylhydrazin behandelt wurde, die Reaktion sowohl in Gegenwart als in An- oder Abwesenheit von Glucose nicht in normaler Weise vor sich geht. Statt des zuerst von Neuberg beschriebenen Osazons, eines gelben, bei 127 bis 130° schmelzenden Körpers, bildete sich eine Verbindung, die in braunen oder grünen Nadeln krystallisierte. Beide Formen schmolzen bei 146 bis 147° und waren anscheinend identisch, aber ihre genaue Konstitutionsformel und Beziehung zu dem wirklichen Osazon konnten noch nicht definitiv aufgeklärt werden.

Eine sorgfältige Wiederholung von Jensens Versuchen sowohl mit reiner wie mit vergorener Glucose führte weder zur Isolierung von Methylphenylglycerosazon noch dieses neuen Gebildes, obgleich winzige, zu solchen Lösungen gesetzte Dioxyacetonmengen leicht durch die Bildung von Krystallen jener neuen Verbindung entdeckt werden konnten. Jensens Behauptungen über das Vorkommen von Dioxyaceton in Glucose und seine Bildung bei Traubenzuckergärung konnten daher nicht bestätigt werden.

Ferner gibt Jensen an, daß wässerige Lösungen von Dioxyaceton, die durch Einwirkung von Wasserstoffperoxyd auf Glycerin in Gegenwart von Eisensulfat gewonnen sind, durch Tierkohle in Kohlensäure und Alkohol gespalten werden. Die von Jensen erhaltenen Mengen dieser Spaltungsprodukte waren jedoch so minimal, daß die Versuche als nicht befriedigend angesehen werden können. Eine unter allen Kautelen vorgenommene Wiederholung derselben mit reinem Dioxyaceton hat zu negativen Resultaten geführt. Von ähnlichen Mißerfolgen waren die Versuche von Karauschanow[1]) und von Euler und Fodor[2]) begleitet, die Beweise für diese Spaltung zu erbringen hofften. Ebensowenig gelang es den letztgenannten Autoren, Dioxyaceton in Glucose aufzufinden.

---

[1]) Karauschanow, Ber. d. Deutsch. bot. Ges. **29**, 322, 1911.
[2]) Euler und Fodor, diese Zeitschr. **36**, 401, 1911.

Versuchsergebnisse.

1. Die Reaktion von Dioxyaceton in verdünnter Lösung mit Methylphenylhydrazin.

Vorversuche haben ergeben, daß die aus sehr verdünnten Lösungen von Dioxyaceton hervorgehende Methylphenylhydrazinverbindung von dem typischen, von Neuberg[1]) beschriebenen Methylphenylosazon verschieden ist. Zu der neuen Verbindung gelangte man wie folgt: 1 g Dioxyaceton wurde in 400 ccm Wasser gelöst und 4 g reines in 40 ccm 50%iger Essigsäure gelöstes Methylphenylhydrazin zugesetzt. Nach Zusatz von 70 ccm Eisessig zu der Mischung füllten wir sie auf 1000 ccm in einer graduierten Stöpselflasche auf und stellten sie bei 24° in den Brutschrank. Nach 24 Stunden hatten sich nadelförmige Krystalle in der Flasche abgeschieden, die nach 3 oder 4 Tagen abfiltriert, gewaschen und getrocknet wurden. Es resultierten ungefähr 1 g bräunlich gefärbte Krystalle, die bei 140° schmolzen und mit Alkohol oder Äther eine dunkelgrüne Lösung ergaben. Nach zweifachem Umkrystallisieren aus 40%igem Alkohol lieferten sie ungefähr 0.5 g hellgrüne Nadeln vom Schmelzpunkt 147 bis 148°. Die Krystalle waren in kaltem Alkohol, Äther, Benzol oder Chloroform mit dunkelgrüner Farbe leicht löslich, in kaltem Wasser gar nicht, in heißem sehr wenig löslich, in heißer verdünnter Säure etwas mehr. Die dabei entstehende rote Lösung wurde durch Alkalizugabe wieder grün.

In einem zweiten Versuche wurden statt 4 g Methylphenylhydrazin 6 g zugesetzt. In diesem Falle bildeten sich 2 g braune Krystalle vom Schmelzpunkt 138°, die beim wiederholten Umkrystallisieren aus 40%igem Alkohol 0.6 g hellgelbe Nadeln, Schmelzpunkt 147°, ergaben. Diese Krystalle lösten sich in denselben Lösungsmitteln wie die grünen, wobei jedesmal eine grüne Färbung entstand. Wenig löslich zeigten sie sich in heißer, verdünnter Säure; die rosa gefärbte Flüssigkeit wurde nach Alkalisierung wieder gelb.

Der Schmelzpunkt einer Mischung von grüner und gelber Modifikation blieb auf dem Werte 146 bis 147°. Es geht hieraus hervor, daß hier eine neue Substanz in 2 Modifikationen

---

[1]) Neuberg, Ber. d. Deutsch. chem. Ges. **35**, 960, 1902.

vorliegt: einer grünen und einer gelben, die gleichen Schmelzpunkt aufweisen.

0.1023 g der Substanz ergaben 0.2601 g CO_2. 0.601 g H_2O.
0.1041 g der Substanz lieferten 18.8 ccm N (20.2°. 737.36 mm).

$$C = 69.4\,\%. \quad H = 6.5\,\%. \quad N = 20.6\,\%.$$

Die Zahlen für Dioxyacetonmethylphenylosazon sind:

$$C = 69.1\,\%. \quad H = 6.4\,\%. \quad N = 19.0\,\%.$$

Die weitere Untersuchung dieser Substanz ist noch im Gange.

Weiter wurden die Bedingungen studiert, unter welchen Neubergs Methylphenylosazon vom Schmelzpunkt 127 bis 130° aus reinem Dioxyaceton entsteht. Neuberg hatte aus Bleiglycerat und Brom bereitete Glycerose bei seinen Versuchen benutzt. Der springende Punkt dabei ist, wie wir fanden, einfach die Konzentration der Lösung. Wenn Mengen von 1 g Dioxyaceton und 4 g Methylphenylhydrazin zu verschiedenen Volumina in Gegenwart von gleich starker Essigsäure (9.6 g auf 100 ccm) verdünnt wurden, so bildete sich bis zu 100 ccm lediglich Neubergs Verbindung; bei 500 ccm und allen größeren Verdünnungen beobachtete man nur die neue, bei 146 bis 147° schmelzende Substanz, während bei Volumina zwischen 100 und 500 ccm die Bildung eines Gemisches beider Substanzen erfolgt.

Sowohl das verwendete Methylphenylhydrazin wie Dioxyaceton waren rein, ersteres ergab mit Fructose reines Methylphenylglucosazon und letzteres mit Phenylhydrazin reines Glycerosazon.

2. Untersuchung von Glucose und die Produkte ihrer Hefegärung auf Dioxyaceton.

Die von Jensen vorgeschriebenen Versuchsbedingungen wurden so genau wie möglich innegehalten, die einzige Abweichung bestand darin, daß obergärige Hefe aus einer englischen Brauerei bei uns zur Verwendung kam.

A. (Glucose allein.)

40 g Glucose von Kahlbaum wurden in 400 ccm Wasser gelöst und 2.5 g in 50 ccm 96%iger Essigsäure gelöstes Methylphenylhydrazin hinzugesetzt; die Mischung wurde auf

500 ccm in einer graduierten Stöpselflasche aufgefüllt und 14 Tage bei 24° im Brutschrank belassen. Nach Ablauf dieser Zeit wurde die Lösung filtriert, der Rückstand ausgewaschen, getrocknet, mit Äther extrahiert und der Auszug verdampft. Es blieb ein glasiges Pulver zurück, das 0,17 g wog und unter dem Mikroskop keine krystallinische Struktur zeigte.

Dieser Versuch wurde mehrere Male wiederholt; in einigen Fällen erfolgte die Lösung des Methylphenylhydrazin in 50%iger Essigsäure, jedoch blieb das Ergebnis all dieser Versuche negativ.

B. (Glucose, Dioxyaceton).

In den folgenden Versuchen wurden kleine Mengen nach der Bertrandschen[1]) Methode bereitetes Dioxyaceton den vergorenen Glucoselösungen zugesetzt.

Versuch A (Glucose allein) wurde wiederholt, nur daß vor Beginn desselben die 40 g Glucose mit 0,5 g Dioxyaceton versetzt wurden. Hier zeigten sich nach 2 Tagen Krystalle in der Lösung, die abfiltriert, gewaschen und getrocknet 0,3 g wogen und bei 130° schmolzen. Sie wurden aus verdünnter Essigsäure umkrystallisiert, wonach 0,15 g hellgrüne Nadeln mit einem Schmelzpunkt von 144° bis 145° sich abschieden. Der Versuch wurde nochmals mit nur 0,1 g Dioxyaceton neben 40 g Glucose ausgeführt; Ausbeute: 0,12 g bei 120° schmelzende Krystalle. Ihnen haftete ein klebriger Sirup an, immerhin war ihre krystallinische Struktur im Mikroskop klar zu erkennen. Um diese Versuche mit Jensens über unvergorene Glucose genau vergleichbar zu gestalten, fügten wir 0,1 g Dioxyaceton zu den 40 g Glucose und überließen die Lösung nach Zusatz von Methylphenylhydrazin 14 Tage im Brutschrank sich selbst. Nach diesem Zeitraum war ein Niederschlag von 0,21 g abgeschieden, der aus denselben mit dem braunen klebrigen Pulver vermischten Krystallen bestand. Die 14tägige Aufbewahrung der Lösung scheint daher auf die Unreinheit des Produktes einen steigernden Einfluß zu haben.

C. Wenn Glucose unter den von Jensen angegebenen Bedingungen durch Hefe in Gärung versetzt wurde, waren die Resultate ganz genau dieselben wie bei reiner Glucose.

---

[1]) Bertrand, Annal. Chem. Phys. 8, 3, 246, 1904.

Es konnte kein krystallinisches Osazon entdeckt werden, doch bildeten sich regelmäßig bei Zusatz von kleinen Dioxyacetonmengen (0,05 g zu 45 g Glucose) Krystalle. Mehrere Versuche wurden mit Natriumsulfat oder Hydroxylaminchlorhydrat nach den Vorschriften von Jensen ausgeführt. Hier folgt die Beschreibung eines der beweiskräftigsten:

90 g Glucose (Kahlbaum) wurden in 900 ccm Wasser gelöst, 450 g $Na_2SO_4 + 10\,H_2O$ und 45 g frische Preßhefe zugegeben und bei Zimmertemperatur 24 Stunden lang stehen gelassen. Hierauf wurde das Gemisch durch ein Berkefeld-Filter filtriert und das erhaltene klare Filtrat in 2 Portionen geteilt.

I. Eine Portion der Lösung wurde mit 2 g Methylphenylhydrazin in 50 ccm 96%iger Essigsäure versetzt, auf 500 ccm aufgefüllt und in einer graduierten Stöpselflasche in einen Brutschrank bei 24° gestellt. Nach Ablauf von 20 Tagen wurde die Lösung filtriert, der Niederschlag gewaschen und getrocknet. Wie zuvor war das einzige Endprodukt eine braune klebrige Masse.

II. Zu der anderen Portion der Lösung wurden noch 0,1 g Dioxyaceton vor der Zugabe von Methylphenylhydrazin gefügt, und nach 20 Tagen wurde die Lösung filtriert. Einige auf dem Filter zurückgebliebene Krystalle waren mit einem braunen Sirup verunreinigt; hiervon wurden sie auf mechanischem Wege getrennt. Sie schmolzen bei 127°. Aus 40%igem Alkohol umkrystallisiert, lieferten sie 0,05 g, bei 144° schmelzende Verbindung.

3. Wirkung von Tierkohle auf Dioxyaceton.

7 g krystallinisches Dioxyaceton wurden in 25 ccm Wasser zur Lösung gebracht und $^1/_2$ Stunde bei 37° in einen Brutschrank gestellt. Auf diese Weise wird das in der Kälte vorhandene dimolekulare Produkt, das durch Tierkohle nicht spaltbar sein soll, in die spaltbare monomolekulare Form zerlegt. Nach Bertrand soll dies fast augenblicklich bei 30° vor sich gehen. Nach Zugabe von 1 g Tierkohle wurde die Flüssigkeit mit Kohlensäure gesättigt und der Kolben mit einem Gärapparat[1]) in Verbindung gesetzt. Es bildete sich keine Spur von Gas, obgleich der Versuch 24 Stunden dauerte.

Bei einer Wiederholung des Versuches entwickelten sich 0,6 ccm CO_2. Auch eine Prüfung auf Alkohol wurde ausgeführt. 1 g Dioxyaceton wurde in 100 ccm Wasser gelöst und 2 Stunden bei 37° im Brutschrank verwahrt. Nach Zu-

[1]) Harden, Thompson und Young, Biochem. Journ. 5, 230, 1910.

satz von 2 g Tierkohle blieb hierauf die Lösung bei Zimmertemperatur 24 Stunden stehen. Nach Ablauf dieser Zeit wurde die Tierkohle abfiltriert, die Lösung destilliert, 50 ccm Destillat aufgefangen und durch nochmalige Destillation auf 30 ccm gebracht. Die Dichte dieses Destillats wurde mittels eines Pyknometers zu 1,000 ermittelt. Zur Kontrolle wurde ein ähnlicher Versuch ohne Zusatz von Tierkohle angestellt; auch in diesem Falle betrug die Dichte des Destillates 1,000.

Unter diesen Bedingungen findet also keine Alkohol- und Kohlensäurebildung aus Dioxyaceton unter Einwirkung von Tierkohle statt.

Zusammenfassung.

1. In verdünnten Lösungen bildet Dioxyaceton bei der Reaktion mit Methylphenylhydrazin eine Substanz, die vom typischen Glycerosemethylphenylosazon verschieden ist, bei 146 bis 147° schmilzt und entweder in gelben oder grünen Nadeln erhalten wird.

2. Dioxyaceton konnte in Traubenzucker weder vor noch nach Vergärung mit englischer obergäriger Hefe unter den von Jensen beschriebenen Bedingungen beobachtet werden.

3. Reines Dioxyaceton wird durch Tierkohle bei 37° nicht in Alkohol und Kohlensäure gespalten.

[*Reprinted from the Journal of Physiology,*
Vol. XLIII. No. 1, 1911.]

ON THE "HEAT COAGULATION" OF PROTEINS. PART II. THE ACTION OF HOT WATER UPON EGG-ALBUMEN AND THE INFLUENCE OF ACID AND SALTS UPON REACTION VELOCITY.

By HARRIETTE CHICK, D.Sc., *Assistant, Lister Institute of Preventive Medicine,* and C. J. MARTIN, M.B., D.Sc., F.R.S., *Director of the Lister Institute of Preventive Medicine.*

In our first communication on this subject(1), we showed that "heat coagulation" was a reaction between proteins and water which progressed in an orderly manner, the reaction velocity being accelerated to an extraordinary degree by raising the temperature or by increasing the concentration of hydrogen-ions in the solution. In the case of hæmoglobin the reaction was found to be of the first order, but with pure crystallized egg-albumen the rate of coagulation diminished more quickly as the reaction progressed than could be accounted for by the diminishing concentration of protein. How far this falling off in rate was due to the observed simultaneous *progressive* diminution in acidity as the protein left the solution, we were unable to ascertain; we contented ourselves with the surmise that it might be so explained, but were unable to express the progress of the reaction by any simple formula.

From an analysis of our results Sutherland(2) arrived at the opinion that the data justify the conclusion that the heat coagulation of egg-albumen is a reaction of the second order, the rate of the reaction at any moment being proportional to the square of the concentration of residual albumen $\left(\frac{-dc}{dt} = kc^2\right.$, or the velocity constant $k = \frac{1}{t}\left(\frac{1}{C_t} - \frac{1}{C_0}\right)$, where C_0 and C_t represent the concentration of unchanged albumen at the beginning and end of the time t). We could not understand how Sutherland had obtained so constant a value for this expression from our experimental results. We had at the time tested our experimental results to see how they might accord with this interpretation,

but with two exceptions (*loc. cit.* Exps. 5 and 6, Table V, p. 419) the constant fell continuously to two-thirds or one-half of its initial value during the progress of the reaction. We have again calculated the constant for the various experiments, and obtained values different from Sutherland's, and on carefully going over his paper find that he has assumed that the reaction was proceeding at constant temperature from the time when the albumen solution was placed in the thermostat. This assumption is unjustified, because by so doing the time, five to ten minutes, taken for the solution to warm up is neglected. Only after this has taken place can the reaction be studied. When this mistake is rectified the figures lend no support to the interpretation that in the case of albumen we have to deal with a reaction of the second order. Further, Sutherland has not considered change in reaction of the solution which is taking place all the time; this is a disturbing factor of sufficient magnitude to invalidate the argument.

Sutherland's second deduction from our observations, viz.: that, over the range studied, the coagulation rate of egg-albumen is also directly proportional to the concentration of hydrogen-ions (as determined in the solution before heating), is subject to the same error, and when this is rectified the proportionality is approximate only over a small interval.

The average velocity, as determined from the time occupied in reducing concentration of albumen from 6 to 3 mgs. per c.c. (*loc. cit.* Tables VI and VII and Fig. 7), when plotted against concentration of hydrogen-ions, gives a curve (Fig. 1) showing that the influence of initial acidity on rate becomes progressively more marked as the former increases.

For example, change in the average velocity, on altering concentration of hydrogen-ions from 25 to 50×10^{-7} normal, was in the proportion of 5·5 to 10·5 or nearly 1 to 2. In more acid solutions a greater proportional change took place, and on increasing concentration of hydrogen-ions from 125 to 250×10^{-7} normal, the average velocities were increased from 28 to 84 or 1 to 3 (see Fig. 1)[1].

We do not think, however, that the exact relation of reaction velocity to hydrogen-ion concentration can be arrived at by such experiments as those published in our previous paper. The conditions are too

[1] We also have evidence that in more acid solution still (hydrogen-ion concentration equal to 4000×10^{-7} normal) an increase of only 15 % in concentration of hydrogen-ions was sufficient to double the average velocity of coagulation.

complicated. Both the concentration of protein and the concentration of acid are changing all the time as the separation of protein from the solution takes with it acid or what amounts to the same thing possibly sets free abase. All we can claim for these experiments is that they show changes in acidity, in the region where concentration of hydrogen-ions is equal to 10^{-5} normal to 10^{-7} normal, to exert a very potent influence upon the reaction rate, which accounts for the old established observation that increase of acidity lowers the "coagulation temperature" of protein solutions.

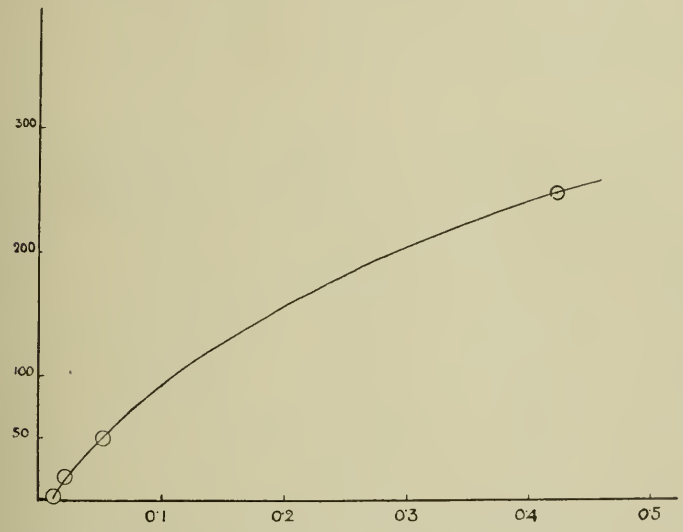


Fig. 1. Relation between average velocity of egg-albumen coagulation (in 1 $\%$ solution) and concentration of hydrogen-ions in the solution.
Ordinates = concentration of hydrogen-ions in terms of normality ($\times 10^7$).
Abscissæ = average velocity of coagulation in mgs. per c.c.

Heat coagulation of egg-albumen a reaction of the first order when precautions are taken to maintain concentration of hydrogen-ions constant.

Enough has been said to show that it is hopeless to attempt to ascertain the nature of the reaction between hot water and egg-albumen unless the concentration of hydrogen-ions can be maintained constant during the observations.

Our first endeavour to arrive at such constancy was to introduce as much as possible of a weakly ionised acid, such as butyric acid, in the

hope that the amount of acid withdrawn during coagulation would not materially diminish the concentration of hydrogen-ions, in other words that the large un-ionised fraction would act as a reservoir of hydrogen-ions. Accordingly an experiment was arranged in which coagulation took place in a concentration of butyric acid equal to $\frac{1}{10}$th normal.

The method of experiment in this and all the subsequent experiments in this paper was exactly similar to that described in our previous paper(1), p. 408. The material used was a solution of pure crystallized egg-albumen prepared according to the method of Hopkins and Pinkus(3) and recrystallized. The 1 % solution of albumen here employed contained 0·26 % of ammonium sulphate. The details of the experiment are given in Table I.

TABLE I. *Coagulation of a* 1 % *solution of crystalline egg-albumen at* 56·2° C. *in presence of excess of butyric acid; initial hydrogen-ion concentration* $= 10^{-3.39}$ *normal* (4070×10^{-7} N), *concentration at* 20 *minutes* $= 10^{-3.47}$ (3370×10^{-7} N) *normal.*

Time, minutes, $= t$	Amount of filtrate analysed, c.c.	Weight of coagulum, gms.	Residual albumen, mgs. per c.c. $= C$	$\frac{1}{t - t_0}(\log C_0 - \log C)$*
0	Control	—	10·100	—
$8 = t_0$	11·2	·0623	$5·563 = C_0$	—
10·1	14·6	·0554	3·795	·0790
12	18·3	·0513	2·803	·0742
15	19·2	·0363	1·891	·0669
20	50	·0634	1·268	·0535

* The values of this expression are in this and other cases calculated with Briggs' logarithms in place of natural logarithms.

The experiment was only partially successful for the concentration of hydrogen-ions was reduced by about 15 % during the progress of the reaction, falling from 4070×10^{-7} normal at the moment of commencing turbidity, to 3370×10^{-7} normal after twenty minutes had elapsed and most of the protein had been precipitated. We therefore tried to ensure constancy of acidity by working in a saturated solution of a very weak acid in presence of undissolved acid. For this purpose we chose boracic acid, which, even at a temperature of 50° C., at which temperature about a 9 % solution is obtained, does not give rise to a concentration of hydrogen-ions too high for our purpose. In a saturated solution at 51° C., the temperature at which the experiment was made, the concentration of hydrogen-ions is rather less than N/1000, ($10^{-3.1}$ normal or $8·0 \times 10^{-4}$ normal).

Precautions were taken to purify the boracic acid used by recrystallization. Excess of the crystals was added to a 1·5 % solution of albumen, warmed up slowly to about 49° C. When the tube containing the solution was placed in the bath at 51° C. a small further excess was added, with the object of supplying a reservoir as the acid was removed. Under these circumstances five minutes was found adequate for the tube and contents to take the temperature of the bath. From time to time samples were removed, cooled, filtered and the residual concentration of albumen determined in the ordinary way.

TABLE II. *Coagulation of a* 1·5 % *solution of crystalline egg-albumen at* 51·1° C. *in presence of saturated boracic acid; concentration of hydrogen-ions constant throughout,* $= 10^{-3\cdot1}$ *normal* $(8000 \times 10^{-7}\ \mathrm{N})$[1].

Time, minutes, $=t$	Amount of filtrate analysed, c.c.	Weight of coagulum, gms.	Residual albumen, mgs. per c.c. $= C$	$\frac{1}{t-t_0}(\log C_0 - \log C)$
$5 = t_0$	12	·1243	$10{\cdot}358 = C_0$	—
15	15	·1306	8·707	·0075
30	19·5	·1332	6·831	·0072
62	28·8	·1098	3·813	·0076
101	30	·0650	2·166	·0071

The details are given in Table II. In the fifth column are given values of the velocity constant, calculated on the assumption that coagulation-rate is proportional only to the protein concentration. A very constant value is obtained, showing that this assumption is true and can be demonstrated if precautions are taken to prevent alteration in concentration of acid. The same result is shown in Fig. 2, where logarithms of concentration of protein as ordinates are plotted against time as abscissae, and the experimental points are seen to lie on a straight line.

The value of this velocity constant in other experiments where no effort was made to keep acidity constant (*loc. cit.* Tables II and V), decreased during the course of the reaction to from 1/2 to 1/7 of its original value. In the experiment with excess of un-ionised butyric acid the decrease in the value of the constant (calculated in the same way) fell to 0·67 of its original value (last column of Table I). The relative change in concentration of hydrogen-ions during this experiment

[1] By matching the tint with methyl-orange given by a saturated solution of boracic acid at 51° C. with that given by a citrate mixture, according to the method of Sörensen[4].

was small, but it occurred at an acidity where small changes in reaction have a very large effect upon coagulation rate.

We therefore conclude that, when freed from disturbance consequent on changing acidity, coagulation of egg-albumen, as we had found to be the case with hæmoglobin, proceeds as a reaction of the first order, coagulation rate at any moment being proportional to concentration of protein.

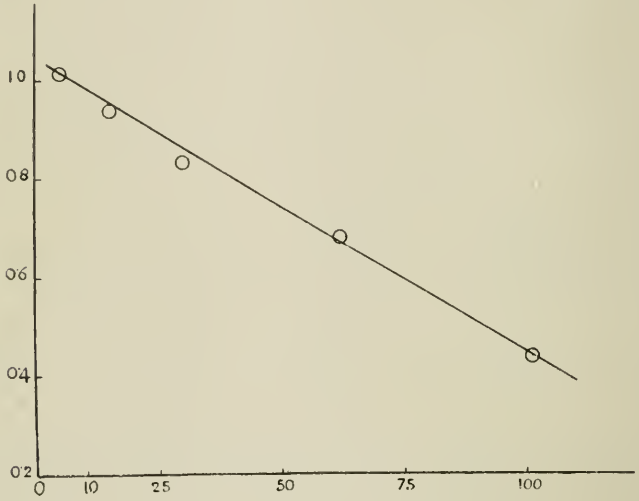


Fig. 2. Coagulation of egg-albumen in presence of saturated boracic acid at 51·1° C. Ordinates = $\log_{10}$ (concentration of residual albumen in mgs. per c.c.). Abscissae = time in minutes.

On the progressive change of acidity which occurs during coagulation.

The following experiments were undertaken with the view of elucidating the more usual and complex case of egg-albumen coagulation where no precautions are taken to maintain a constant reaction throughout the process. The diminution in concentration of hydrogen-ions which takes place during coagulation was studied by measurements made after successive intervals of time during the process, and the corresponding concentration of residual albumen was also ascertained.

The details of two such experiments, 1 and 2, are given in Tables III and IV, Exp. 1 made at 68·7° C. with an initial acidity of 135×10^{-7} normal, Exp. 2 at 71° C. in the presence of a concentration of sodium

chloride equal to normal and an initial acidity of 45×10^{-7} normal. Both solutions contained 0·26 % ammonium sulphate[1].

In both experiments free acid was progressively removed from the solution together with the protein. The coagulation of unit weight of protein does not remove quite the same amount of free acid throughout the experiment, but a less amount as coagulation proceeds. This might have been anticipated from observations in our previous paper(a), (p. 427) in which the amount of acid fixed by coagulation of one gram of protein varied from $0{\cdot}5 \times 10^{-7}$ to 459×10^{-7} equivalents, when the initial free acid in the solution was changed from 1×10^{-7} to $8590 \times 10^{-7} \times$ normal.

A chance relationship between residual protein and hydrogen-ion concentration emerges from an analysis of the data given in Tables III and IV. If these be plotted one against the other, the points fall upon a straight line or very shallow curve, so that over the small range of acidity of these two experiments the two are nearly proportional.

Further, within a portion of the range of acidity of most of our experiments on reaction-rate, an approximate proportionality also exists between velocity of reaction and concentration of hydrogen-ions, as may be seen from Fig. 1 above, in which the portion of the curve between the ordinates 10^{-5} and $10^{-7} \times$ normal is, within the error of experiment, linear.

These two results explain the fact that coagulation of egg-albumen, without compensation for varying acidity, may occasionally approximate to a reaction of the second order, see column 6, Tables III and IV. This is, however, merely coincidental for, as we have shown above, the velocity of reaction uncomplicated by changes in acidity is simply proportional to protein concentration.

This spurious relation of rate to square of protein-concentration is due to the facts to which attention has just been drawn, that, over a certain range of acidity, rate of reaction is nearly proportional to hydrogen-ion concentration, which in its turn is roughly proportional

[1] When the very slight acidity (10^{-5} to $10^{-7} \times$ N) obtaining in these experiments is taken into account, an error is apparent owing to neutralisation of an appreciable amount of the acid by the soluble alkali contained in the glass vessel in which the coagulation was carried out. A control experiment was made at 71° C. with a similar glass tube containing a solution of corresponding low acidity ($33 \times 10^{-7} \times$ N), but containing no protein, and this was shown to be the case, the acidity being reduced to $11{\cdot}2 \times 10^{-7}$ N. Most of the decrease, however, (to $14{\cdot}5 \times 10^{-7}$ N) took place during the first 10 minutes, and since in experiments 1 and 2, Tables III and IV, the determinations of concentration of acidity and protein began only after 10 minutes had elapsed, the error is not very great. It would, however, have been better if the experiments had been carried out in quartz vessels.

to the concentration of protein remaining. Under these conditions, a reaction of the first order simulates one in which the reaction velocity at any moment is proportional to the square of the protein concentration, because owing to the nearly linear relationships happening to obtain between concentration of protein and hydrogen-ions on the one hand, and hydrogen-ions and reaction-velocity on the other, it is as if the concentration of protein affected the rate of reaction twice over.

In the two experiments under consideration the value of the constant $k = \frac{1}{t}\left(\frac{1}{C} - \frac{1}{C_0}\right)$, where C_0 = the initial concentration of protein, and C the concentration after time t, falls off only slightly as the reaction proceeds. In most of the experiments set out in our previous

TABLE III. Exp. I. *Progressive change in acidity during coagulation of a* 1 % *solution of crystalline egg-albumen at* 68·7° C.

Time, minutes, $= t$	Amount of filtrate analysed, c.c.	Weight of coagulum, gms.	Residual albumen, mgs. per c.c. $= C$	$\frac{1}{t - t_0}(\log C_0 - \log C)$	$\frac{1}{t - t_0}\left(\frac{1}{C} - \frac{1}{C_0}\right)$	Concentration of H^+ ions in terms of normality $\times 10^7$
0	Control	—	9·750	—	—	135 N
$10 = t_0$	20	·1560	$7·800 = C_0$	—	—	79·3
14	28	·1744	6·228	·024	·00807	57·4
20	41·9	·2046	4·887	·020	·00764	42·4
37	44·4	·1360	3·066	·015	·00740	19·6

TABLE IV. Exp. II. *Progressive change in acidity during coagulation of a* 1 % *solution of crystalline egg-albumen at* 71° C., *in presence of a concentration of* NaCl = *normal solution.*

Time, minutes, $= t$	Amount of filtrate analysed, c.c.	Weight of coagulum, gms.	Residual albumen, mgs. per c.c. $= C$	$\frac{1}{t - t_0}(\log C_0 - \log C)$	$\frac{1}{t - t_0}\left(\frac{1}{C} - \frac{1}{C_0}\right)$	Concentration of H^+ ions in terms of normality $\times 10^7$
0	Control	—	9·750	—	—	45·0
$10 = t_0$	19	·1785	$9·395 = C_0$	—	—	33·5
30	21·1	·1511	7·161	·0059	·00166	18·2
100	36·3	·1493	4·113	·0039	·00152	9·53
310	38	·0716	1·884	·0023	·00145	2·07

paper(1), (Tables II and V), the constant decreased in value to a much greater extent.

In some cases in presence of more concentrated salts, (see Table VIII, p. 19), a very perfect agreement happened to be maintained and coagulation proceeded in extraordinary agreement with the progress of a reaction of the second order.

The union of egg-albumen with acid (1) *in the cold, and* (2) *on coagulation by hot water.*

It is an old observation that when protein is added to acid a diminution of acidity and of conductivity takes place, and that the solutions become still less acid after coagulation by heat has occurred. Details of some observations upon this subject and some fresh experiments made by ourselves were given in our previous paper (*loc. cit.* Tables VII and IX). These experiments we have endeavoured to complete in the present instance.

(1) *Fixation of acid by egg-albumen in the cold.* That proteins form compounds with acids and bases was pointed out as long ago as 1866 by Platner(5), since when the reaction between proteins and acids has been studied by a variety of methods. As this only indirectly concerns us, and as the literature has recently been collected by Brailsford Robertson(6), it is unnecessary to refer to it in detail.

The observations of Bugarszky and Liebermann(7) and Moore and Bigland(8) on the equilibrium of proteins with acid, however, call for mention as they bear directly upon the point under consideration. Bugarszky and Liebermann determined, by means of the concentration cell, the fall in acidity when varying quantities of protein were added to 0·05 N. HCl. Moore and Bigland placed a definite amount of protein solution and acid in a dialyser surrounded by a known volume of water and titrated the acid in the water outside the membrane after a sufficient time had elapsed for equilibrium. Both sets of observers found that the amount of acid taken up by protein varies with the concentration of free acid, but it is to be regretted that in neither case were the experiments made with pure proteins. These experiments show that the acid combined reaches a maximum above which practically no more is taken up.

The amount of acid fixed in the cold by a constant weight of pure egg-albumen in concentration of hydrogen-ions from 1 to 250×10^{-7} normal was incidentally determined by us in our previous experiments(1)

(p. 423). These observations have now been extended nearly to the point where the protein begins to suffer attack by the acid. Measured amounts of standard H_2SO_4 were added to a fixed quantity of a 1 % solution of pure crystallized egg-albumen; water was added to make each solution up to a constant volume, and about two hours allowed for equilibrium to be reached.

Following the method of Bugarszky and Liebermann, the actual free acid was deduced from the observed concentration of hydrogen-ions in the solution, as determined electrically by means of a hydrogen concentration cell. The method was exactly the same as that already described in detail(1) (p. 422). As H_2SO_4 is not completely ionised in the strengths with which we were working, we had to calculate the concentrations of free H_2SO_4 from our determinations of the hydrogen-ions present. For this purpose we took the values for the dissociation of this acid in different strengths arrived at by Kohlrausch[1] from conductivity experiments. We plotted dissociated acid obtained from Kohlrausch's determinations against total concentration of acid and drew a smoothed curve. From the curve we read off the concentration of $\frac{1}{2}$ H_2SO_4 corresponding to the particular hydrogen-ion concentration required.

The results of the experiments are given in Table V and graphically represented in Fig. 3, where equivalents of acid fixed per gram protein ($\times 10^3$) are plotted against the final concentration of acid ($\times 10^3$). The amount of acid combined rises rapidly at first, then more slowly, and from the curve, is apparently approaching its maximum in a concentration of 0·03 normal $\frac{1}{2}$ H_2SO_4.

From the tables given by Bugarszky and Liebermann the HCl fixed per gram of mixed egg-white proteins in equilibrium with different concentrations of free acid can be obtained. These values do not coincide with our own for equivalents of H_2SO_4 and pure egg-albumen but are considerably less[2]. The general form of the curve obtained by plotting these values is, however, the same.

The reversibility of this action between protein and acid was demonstrated by Moore and Bigland(8) (p. 40), and confirmed in the present instance. Two experiments were made (Nos. 9 and 10) to correspond to Experiments 5 and 6 in Table V; the solutions were made up to contain twice the concentration of acid and twice the concentration of protein that was present in the earlier experiments.

[1] Landolt, Börnstein and Meyerhoffer. *Physikal.-chem. Tabellen*, No. 229 *b* Berlin, 1905.

[2] H_2SO_4 probably combines to some extent as $R\text{-}NH_2HSO_4$.

After contact for two hours, the solutions were both diluted twice with distilled water, so that they then became identical in every respect with the solutions of Experiments 5 and 6. The final concentration of hydrogen-ions was then measured in solutions 9*a* and 10*a*, and agreed very nearly in each case with that of the corresponding previous Experiments 5 and 6. This showed that the absorption of acid in the 2% solution was reversible and that readjustment took place on dilution.

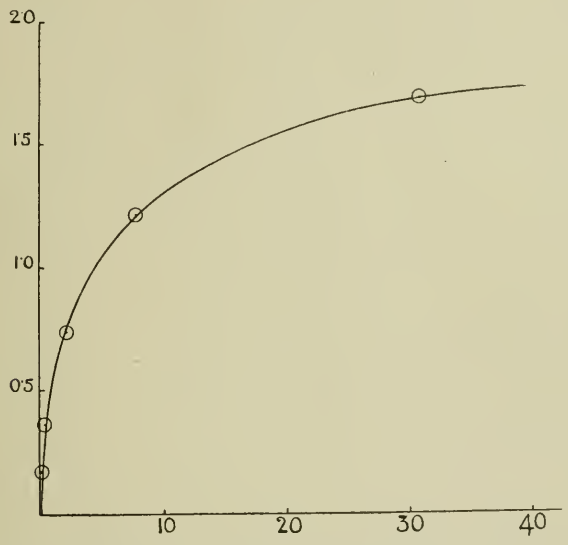


Fig. 3. Fixation of acid (H_2SO_4) by 0·95 % solution of egg-albumen at room temperature.
Ordinates = equivalents of acid fixed per 0·95 gram protein ($\times 10^3$).
Abscissæ = final concentration of acid in terms of normality ($\times 10^3$).

(2) *Further fixation of acid on coagulation.* Earlier observations on the diminution of the acidity of protein solutions on coagulation were referred to in our previous paper, where (p. 427) we also recorded the amount of free acid fixed in our own experiments by one gram of egg-albumen in solutions containing varying concentration of hydrogen-ions. Recently Sörensen and Jürgensen(9) have also made similar measurements, and further shown that starting with amounts of acid giving the same hydrogen-ion concentration, the fall in concentration of hydrogen-ions is less with a weak than with a strong acid. The smaller fall in hydrogen-ion concentration in the case of the weaker acid might have been expected, as the un-ionised fraction serves as a reservoir.

TABLE V. *Fixation of acid by crystallised egg-albumen* (0·95 % *solution*) *at room temperature.*

Total volume in each case = 52·5 c.c.

Exp.	Amount of 1 % albumen solution taken, c.c.	Amt. of N. $\frac{1}{2}(H_2SO_4)$ (or equivalent) added, c.c.	Concentration of free acid ($\frac{1}{2}H_2SO_4$) which would have resulted in absence of protein, in terms of normality, $\times 10^3$	Actual final concentration of H^+, in terms of normality, $\times 10^3$	Calculated[1] final concentration of $\frac{1}{2}H_2SO_4$, in terms of normality, $\times 10^3$	Equivalents of acid fixed per 0·95 gm. protein, $\times 10^3$
1 *a*	50	(0·1 c.c. N/1 AmOH)	—	·00094 N	—	—
1	50	0	—	·0154	—	—
2	50	0·1	1·905 N	·071	·071	·183
3	50	0·2	3·81	·195	·195	·361
4	50	0·5	9·52	2·04	2·13	·739
5	50	1·0	19·05	6·78	7·86	1·119
6	50	2·5	47·62	22·50	30·66	1·696
	2 % solution					
9	50	2·0	38·10	11·12	13·55	—
9 *a*	Exp. 9, diluted twice with distilled water		19·05	6·55	7·55	1·150
10	50	5·0	95·25	38·91	5·80	—
10 *a*	Exp. 10, diluted twice with distilled water		47·62	22·81	31·16	1·646

In their single experiment bearing on this point, which appears to have been made with undialysed egg-white solution, they found the total acid neutralised per gram of protein was approximately the same when they started with such amounts of hydrochloric, lactic and acetic acids as to afford the same hydrogen-ion concentration (*loc. cit.* Table XII, p. 427). We have not obtained this result with pure egg-albumen, but as was found to be the case in our earlier experiments, the amount of acid which disappears per gram of egg-albumen coagulated is primarily a function of the hydrogen-ion concentration.

A comparison of Exps. 1 and 2, and 3 and 4 in Table VI below shows that the amount removed is dependent upon hydrogen-ion concentration whether the particular acid be a strong or a weak one. With a concentration of hydrogen-ions equal to about $10^{-4\cdot1}$ normal the amount withdrawn was greater with acetic than with hydrochloric acid, with a concentration equal to about $10^{-2\cdot6}$ normal the reverse was true, but

[1] From Kohlrausch's determinations of conductivity, Landolt, Börnstein and Meyerhoffer. *Physikal.-chem. Tabellen*, No. 229 *b*. Berlin, 1905.

it will be seen that whereas the removal of the acid in the case of hydrochloric produced a considerable fall in acidity, in the case of acetic this effect was much less.

TABLE VI. *Disappearance of acid added and alteration in hydrogen-ion concentration on coagulation of a* 1·25 % *solution of egg-albumen containing* 0·36 % Na_2SO_4 *and previously acidified with hydrochloric and acetic acid respectively.*

Exp.	Acid added	Amount of acid added in 100 c.c., no. of c.c. N/10 (or equivalent)	Amount of acid remaining in 100 c.c. filtrate after coagulation in terms of no. of c.c. N/10 NaOH (or equiv.) required to neutralise to phenolphthalein	Total acid fixed in terms of equivalents acid fixed per gm. protein $\times 10^3$.	Concentration of H^+ ions in terms of normality		Additional[1] acid fixed on coagulation in terms of equivs. per gm protein $\times 10^3$
					In original solution after addition of acid in the cold	In filtrate after coagulation by heating	
1	HCl	3	0·2	0·22	$10^{-4.19}$ normal (650×10^{-7} N)	$10^{-4.71}$ normal (195×10^{-7} N)	·0036
2	,,	13·65	2·78	0·87	$10^{-2.61}$,, (24600×10^{-7} N)	$10^{-2.72}$,, (19500×10^{-7} N)	·041
3	$C_2H_4O_2$	13·65	9·12	0·36	$10^{-4.05}$,, (892×10^{-7} N)	$10^{-4.23}$,, (590×10^{-7} N)	—
4	,,	997·1	990·6	0·52	$10^{-2.63}$,, (23400×10^{-7} N)	$10^{-2.65}$,, (22500×10^{-7} N)	—

Interpretation of the fixation of acid on coagulation.

The amphoteric character of proteins was explained by Emil Fischer by their polypeptide constitution. As complex amino-acids they form salts with acids and alkalies, the acid joining on to the NH_2 group with change of the valency of nitrogen from triad to pentad, and the base displacing the H in the carboxyl. The salts with acids hydrolyse in water so that they are only stable in the presence of a small excess of acid. A further source of acidity is the dissociation of the hydrogen of the carboxyl group.

When a protein salt with an acid, *e.g.* HCl, is dissolved in water a certain amount of the acid is split off by hydrolysis until equilibrium is attained. If such solution be dialysed the free acid passes through the membrane and a further hydrolysis occurs. In time the whole of

[1] These amounts were calculated from the determinations of H^+ ion concentration, which in the case of HCl are proportional to the concentration of acid.

the HCl is removed and a chlorine-free solution is obtained which possesses a faint acidity, approximately 10^{-5} normal which apparently represents the electrolytic dissociation of the protein.

If such a solution be heated to a sufficient extent to precipitate the egg-albumen, *pari passu* with the separation of the protein, the acidity, as we have shown above, diminishes.

The disappearance of this small amount of acidity could be adequately explained on the supposition of electrolytic dissociation of the protein, for in this case the removal of the protein would also remove the hydrogen-ions. This explanation is the one adopted by Sörensen and Jürgensen(9) to explain their observation that if to the originally alkaline egg white solution HCl be added until the acidity equals that of a concentration of hydrogen-ions of about 10^{-5} N and then coagulation takes place, the acidity diminishes, but, nevertheless, the whole of the chlorine is found in the filtrate. They conclude from this that the HCl added to arrive at the iso-electric point was entirely occupied in the neutralisation of some base contained in the originally alkaline egg white solution, and none was available to form hydrochloride with the amino-group of the albumen. If their supposition were correct the solution would contain only the hydrogen salt of the protein, and chlorine combined with some base originally attached to the protein. In such a solution the whole acidity might, as they point out, be due to the electrolytic dissociation of the protein which is behaving as a weak acid.

We tried to repeat these experiments of Sörensen and Jürgensen and to extend them so as to include the case of more acid solutions where salt formation between protein and acid undoubtedly exists. This seemed to us important because from their results it might be inferred that in the coagulation of solutions containing salts of proteids with acids the latter did not leave the solution with the precipitated protein. We found, however, that a solution of purified crystals of egg-albumen which has been dialysed until it is free of Am_2SO_4 does not form a precipitate on heating in the presence of HCl. Precipitation occurs, however, if neutral salts are added, but the amount of a chloride which is necessary to effect agglutination makes the total chlorides to be estimated in the filtrate so great that the determination of any loss of chlorine by the fixation of HCl is rendered uncertain. A very small addition of a sulphate of sodium, potassium or ammonium is able to bring about complete separation of the heated protein in a particulate form. In the presence of 0·014 N. HCl and 0·36 % Na_2SO_4 we found that although acid disappeared on coagulation the whole or

nearly the whole of the chlorine remained in the filtrate. On subsequent experimentation we ascertained, however, that a corresponding quantity of SO_4 had combined with the coagulum. We therefore had recourse to H_2SO_4 for acidifying the solution of egg-albumen crystals and a small quantity of Na_2SO_4 was added to facilitate separation of the coagulum. Only a small amount of this salt is required because SO_4 assists the agglutination of denaturated egg-albumen much more powerfully than Cl.

In the following experiment, the precise details of which are set out in Table VII, 500 c.c. of 1 % egg-albumen was made up, containing an amount of acid which, in the absence of protein, would have made the solution equal to 0·05 N ($\frac{1}{2}$ H_2SO_4). It also contained about 0·1 % of Na_2SO_4. The whole was placed in a Jena flask and heated with a reflux condenser in a bath of boiling water for 30 minutes. At the end of that time it was cooled down and filtered with precautions to avoid evaporation. Portions of the filtrate were taken for the estimation of total acid by titration with $\frac{1}{50}$th N. NaOH and phenolphthalein as indicator, and for the gravimetric determination of total sulphates.

TABLE VII. *Showing that the protein-acid-salt formed on addition of* H_2SO_4 *to a solution of pure crystalline egg-albumen is precipitated as such on coagulation by hot water.*

Material	Amount of acid, in terms of N/10, $\frac{1}{2}$ H_2SO_4, added in 100 c.c. of the solution, c.c.	Amount of Na_2SO_4 in terms of N/10, Na_2SO_4, added in 100 c.c. of the solution, c.c.	Total amount of SO_4, in terms of N/10, $\frac{1}{2}$ SO_4, added in 100 c.c. of the solution, c.c.	Amount of acid, in terms of c.c. N/10 NaOH, added for neutralisation to phenolphthalein, remaining in 100 c.c. filtrate after coagulation, c.c.	Total amount of SO_4, in terms of c.c. N/10, $\frac{1}{2}$ SO_4, present in 100 c.c. filtrate, c.c.	Equivalents of acid fixed per gram protein, $\times 10^5$	Equivalents of $\frac{1}{2}$ SO_4 fixed per gram protein, $\times 10^5$
1 % sol. egg-alb.	20	14·26	34·26	9·64	25·36	1·04	0·89

From Table VII it will be seen that 1 gram of egg-albumen on coagulation in 1 % solution had removed 0·00105 equivalent of acid and about 10 % less of SO_4.

It is clear, therefore, that on coagulation of egg-albumen in more acid solutions the bulk of the acid leaves the solution with the protein. In other words the salt is precipitated as such.

The want of agreement between disappearance of acid and sulphate indicates that some base other than protein has combined with a

portion of the acid. The possibility of a trace of ammonia or di-amino-acid being split off during coagulation was considered, but excluded as the filtrate was found to be nitrogen-free. The only other sources from which a base could be derived are the ash of the protein and alkali from the glass of the vessel in which the protein solution was heated. The ash in our solution of protein was only 0·0016 gr. per gram of egg-albumen; this, even if all composed of MgO or CaO, is a little short of the amount required for combination with the small excess of SO_4 found in the filtrate. On the other hand it is unlikely that such an amount of alkali could be dissolved out of the glass of a Jena flask during 30 minutes' heating at 100° C.

We propose to ascertain for certain whether the small amount of ash contained in our egg-albumen is indeed removed by boiling in this concentration of acid.

Another explanation of the loss of acidity on coagulation is that CO_2 is driven off by the heating. This view, which was formulated by Michaelis and Rona[10] is quite inadequate to explain the large amount of acid disappearing from the more acid solutions of pure egg-albumen we employed, and the progressive nature of the disappearance, for it must be remembered that we were working with crystallized egg-albumen and not with diluted serum or egg-white. Moreover, in determining the hydrogen-ion concentration hydrogen was passed through the cell until a constant reading was obtained whereby CO_2 would be displaced. It is not possible to work with serum proteins in such acid solution as we employed with egg-albumen, owing to failure of agglutination, but there is little doubt that our results could be applied to serum proteids.

Sörensen and Jürgensen[9] (p. 424) have also replied to this theory of Michaelis and Rona by showing that a diminution in hydrogen-ion concentration took place in solutions of egg-white and blood-serum where all carbon-dioxide had been previously driven off by passing through a continuous stream of hydrogen in strongly acid solutions. They also showed that no extra carbon-dioxide was produced during coagulation.

Our conclusions regarding the relation of acid and protein during heat coagulation of pure crystalline egg-albumen may be summarised as follows.

(1) The amount of acid combined as protein-acid-salts, when acid is added to a solution of protein, depends on the concentration of free acid. Conversely, these salts undergo hydrolysis which accounts for the free acid always present in such solution: *e.g.* with HCl.

$$\text{Prot.} - \text{HCl} \rightleftharpoons \text{Prot.} + \text{H}^+ + \text{Cl}^-.$$

(2) On treatment by hot water the protein-acid-salts are precipitated as such; the percentage of acid combined with them being dependent on the acidity of the solution at the time of coagulation.

(3) The interaction between egg-albumen and hot water, if acidity be kept constant, proceeds as a reaction of the first order.

(4) The velocity of the reaction is conditioned by the acidity (hydrogen-ion concentration) of the solution.

(5) Unless precautions be taken to maintain the acidity of the solution at a constant level, the concentration of free acid progressively diminishes as the protein is precipitated.

(6) The conclusions embodied in (3), (4) and (5) explain why under ordinary circumstances the reaction between egg-albumen and hot water appears to be of a more complicated nature.

(7) From (2) combined with (4), it appears that the original protein is acted upon by hot water less readily than protein-acid-salts, and further that these salts suffer attack at a rate which is dependent upon the amount of acid they contain[1]. If this is so, a satisfactory explanation of the progressive diminution of acidity during coagulation is forthcoming, as the separation from the solution of protein-acid-salt will disturb the equilibrium between hydrolysed and unhydrolysed salt. Some portion of the free acid and free protein will therefore combine. This phenomenon will be progressive.

Effect of salts upon the velocity of "heat coagulation" of proteins.

The effect of salts upon "coagulation temperature" has been studied by K. V. Starke(11), Haycraft and Duggan(12), Osborne and Campbell(13), Joh. Starke(14), Pauli and Handovsky(15) and others. All these workers found that the temperature at which precipitation was first observed was raised with increasing concentration of salts.

A higher "coagulation temperature" is, as was shown in our previous paper, an indication of the diminution of "coagulation-rate" at constant temperature.

In our experiments we studied coagulation-rate by the same method as before. Solutions containing 1 % of albumen were made by dilution from a stock solution of egg-albumen crystals. The stock albumen solution contained Am_2SO_4 so that the 1 % solution of egg-albumen had in addition to protein 0·26 % of the salt. It was not found necessary to get rid of this small amount of Am_2SO_4 by dialysis. The rate of coagulation at 70·9° C. was measured in the control solution and also in similar solutions containing in addition various quantities of sodium chloride and extra ammonium sulphate. In the case of the stronger salt solutions the time necessary for the experiments was so long that precautions had to be taken to avoid errors due to evaporation of the

[1] A possible catalytic influence of the hydrogen-ions cannot be excluded.

protein solution. This was successfully done by using a rubber cork and by filling a mixture of vaseline and paraffin wax into the glass bearing in the cork through which the stirrer passed. Under these conditions the loss of water was negligible.

The coagulation-rate was measured up to a concentration of three times normal (equivalents) in the case of ammonium sulphate and of sodium chloride. It was not possible to work with higher concentrations of salt as the rate of coagulation became too slow to study conveniently at

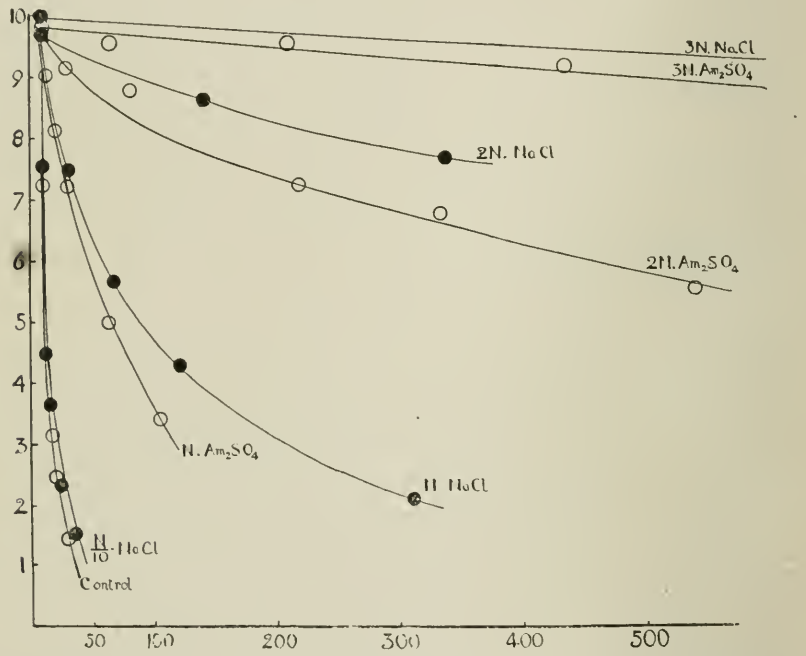


Fig. 4. Effect of addition of salt, NaCl and Am_2SO_4, in various concentrations, upon coagulation rate of 1 % solution of egg-albumen at 70·9° C.
—•— Experiments with NaCl. —o— Experiments with Am_2SO_4.
Ordinates = concentration of residual albumen in mgrs. per c.c.
Abscissæ = time in minutes.

70·9° C., the temperature of experiment. The details of our results are set forth in Table VIII below and are also graphically represented in Fig. 4, where the concentration of residual albumen in the different experiments has been plotted against time.

It will be seen that the presence of salts greatly diminishes coagulation-rate. From the smoothed curves it appears that in a concentration of NaCl equal to normal the rate is only $\frac{1}{16}$th, and in twice normal

TABLE VIII. *Coagulation of* 1 % *solution of pure crystalline egg-albumen, with original salt content* (= 0·26 % Am_2SO_4) *and after addition of* NaCl *and* Am_2SO_4 *in varying concentration at* 70·9° C.

Exp.	Salt added	Concentration of salt in terms of normality	Time, minutes = t	Amount of filtrate analysed, c.c.	Weight of coagulum, grs.	Concentration of residual albumen, mgrs. per c.c. = C	$\frac{1}{C}$	$\frac{1}{t_n - t}\left(\frac{1}{C_n} - \frac{1}{C}\right)$*
1	Control	0	10	8·7	·0455	5·231	·191	·0242
			12	14	·0622	4·443	·225	·0250
			16·17	20	·0630	3·150	·318	·0258
			20	20	·0492	2·460	·407	·0268
			$30 = t_n$	50	·0742 = C_n	1·484	·675	—
							Mean	·0250
2	NaCl	0·1 N	10	10	·0556	5·560	·180	·0191
			12	15·4	·0690	4·480	·223	·0189
			15	35·3	·1290	3·655	·273	·0192
			23	26·4	·0613	2·322	·431	·0191
			$25 = t_n$	26·9	·0409 = C_n	1·520	·658	—
							Mean	·0191
3	NaCl	N	10	12·5	·1214	9·711	·103	·00129
			31	15·5	·1160	7·485	·134	·00127
			67	20	·1130	5·650	·177	·00128
			121	23·3	·0964	4·137	·242	·00130
			$311 = t_n$	32·5	·0664 = C_n	2·043	·490	—
							Mean	·00128
4	NaCl	2 N	10	11·1	·1074	9·676	·103	·0000948
			142	12	·1034	8·617	·116	·0000923
			$337 = t_n$	15	·1142 = C_n	7·614	·134	—
							Mean	·0000935
5	NaCl	3 N	10	Control	—	10·000	·100	·0000143
			940	15	·1258	8·388	·119	·0000115
			1420	18	·1495	8·306	·120	·0000143
			2393	20	·1501	7·505	·133	·0000158
			$3025 = t_n$	21	·1466 = C_n	6·982	·143	—
							Mean	·0000140
6	Am_2SO_4	1·03 N	13	11	·0992	9·018	·111	·00199
			20	20	·1628	8·140	·123	·00201
			30	19·5	·1407	7·215	·139	·00207
			63	22·3	·1113	4·992	·200	·00224
			$105 = t_n$	46	·1565 = C_n	3·403	·294	—
							Mean	·00208
7	Am_2SO_4	2·03 N	10	8·6	·0843	9·803	·100	·000154
			30	15·5	·1421	9·170	·109	·000143
			81	10	·0877	8·770	·114	·000148
			219	21·5	·1549	7·205	·139	·000134
			335	22·4	·1504	6·716	·149	·000160
			$541 = t_n$	26·4	·1450 = C_n	5·493	·182	—
							Mean	·000148
8	Am_2SO_4	3·03 N	10	10	·0966	9·660	·103	·00000412
			66	11·3	·1078	9·540	·105	·00000412
			210	16·2	·1542	9·519	·105	·00000438
			435	16·3	·1485	9·110	·110	·00000461
			1055	24·2	·1892	7·819	·128	·00000535
			$2495 = t_n$	35	·1694 = C_n	4·841	·205	—
							Mean	·00000452

* The values of the constant are calculated using the *last* determination of albumen concentration as standard, owing to the greater accuracy, as regards time, with which these samples can be taken in the quick experiments.

only $\frac{1}{34}$th of that in the control. The effect of Am_2SO_4 was somewhat less. This effect of salts is not due to any influence on the second phase of coagulation, viz. the separation of the altered protein in the particulate form, because in the samples drawn this was completed so that all filtered quickly and well, affording clear filtrates in which no further precipitation occurred.

In the last column of Table VIII are given the values of

$$\frac{1}{t_n - t}\left(\frac{1}{C_n} - \frac{1}{C_t}\right)^1,$$

where C_n the final concentration and C the concentration after time t has elapsed. Although the values show a satisfactory constancy, for reasons stated above, we consider this approximation to a reaction of the second order (the reaction velocity at any moment being proportional to the square of the concentration of residual protein at that moment) is a mere coincidence. This coincidence has, however, proved convenient, as it enables the effect of salts to be quantitatively expressed by comparing the velocity constants characteristic of each experiment.

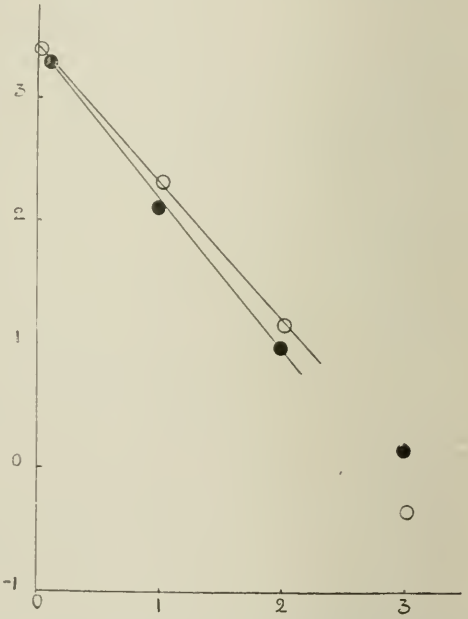


Fig. 5. Relation between the concentration of salt and the coagulation-rate as measured by the velocity constant.

Experiments with NaCl •; experiments with Am_2SO_4 o.
Ordinates = logarithms_{10} (velocity constant $\times 10^5$).
Abscissæ = concentration of salt in terms of normality.

When we plotted the logarithms of the velocity constants obtained for different concentrations of the same salt against the reciprocal of the concentrations of salt, between the limits 0 and twice normal, the points were very nearly in a straight line (Fig. 5), showing that as

[1] See footnote to Table VIII.

the concentration of salt in the solution is increased arithmetically the velocity of coagulation diminished geometrically.

This relation, speculations as to the significance of which appear below, is maintained up to a concentration of twice normal NaCl and Am_2SO_4, but the observation with three times normal salt indicates that with stronger solution the effect of NaCl and Am_2SO_4 becomes slightly less and slightly more[1] potent respectively.

This great retardation of the rate of denaturation by neutral salts becomes of practical significance in the estimation of proteins by the method of Devoto(06). Two hours' heating on a water bath at 100° C. does not complete the process in the presence of saturated Am_2SO_4. The efficacy of Hopkins'(07) modification is due to the subsequent heating in dilute salt solution. The difficulty can also be got over by heating for 5 minutes at 110° C. in an autoclave.

The effect of the addition of neutral salts upon the hydrogen-ion concentration of protein solutions. It occurred to us that the effect of neutral salts in slowing reaction-rate might be, in whole or in part, due to change in the hydrogen-ion concentration of the protein solutions. We, therefore, determined by the electrical method the concentration of hydrogen-ions in our egg-albumen solution, and the same made up to contain a concentration of NaCl varying between $\frac{1}{10}$th normal and twice normal. The results are given in Table IX, and show that acidity is indeed progressively lowered with each increase in concentration of salt. The addition of an amount of salt to make the

TABLE IX. *Effect of addition of various amounts of* NaCl *upon hydrogen-ion concentration and coagulation velocity of* 1 % *egg-albumen solution at* 70·9° C.

Concentration of NaCl in terms of normality	H^+ concentration, in terms of normality	Coagulation velocity as measured by the velocity constants (from Table VIII)
0	$10^{-4.88}$ normal (132×10^{-7} N)	·0250
0·1 N	$10^{-4.88}$,, (132 ,,)	·0191
1 N	$10^{-5.30}$,, (50 ,,)	·00128
2 N	$10^{-5.73}$,, (19 ,,)	·0000935

[1] Pauli and Handovsky(15), working with thoroughly dialysed ox-serum, found that the effect of ammonium sulphate in hindering coagulation-rate (as measured by determining "coagulation temperature") was reversed at a concentration between 2× and 3× normal. In the above experiments we find that the rate of lessening coagulation velocity is on the contrary increased. Further experiments with ammonium sulphate and a solution of pure egg-albumen showed a progressive slowing of coagulation-rate up to the point where precipitation was imminent. We have not made experiments with serum.

solution = N/10 had no appreciable effect, but in normal solution of NaCl the hydrogen-ion concentration was less than 1/2, and in twice normal solution was 1/7th of that of the control solution to which no salt had been added. The most obvious explanation of this action is that in presence of sufficient amount of salt the hydrolysis of the protein-acid-salt is hindered.

This effect of salt in lessening the acidity of solutions containing protein is inconsistent with the conclusions of other observers, derived from indirect methods of measuring hydrogen-ion concentration. Pauli and Handovsky[15], using indicators, found that adding salts to an acidified solution of ox-serum resulted in an increased acidity. Hardy[19] found the same result with salt and "acid albumen."

These observers appear to have been misled by their indicators. Pauli and Handovsky's estimations of acidity were made by comparison of the colour given with indicators, such as methyl-orange and alizarine. The colour of indicators, particularly that of methyl-orange, has since been shown by Sörensen[4] (p. 240) to be modified by the presence of any considerable amount of protein. Michaelis and Rona[20] have found that the tint of an indicator in a solution having a particular hydrogen-ion concentration may also be influenced by the presence of salts, although the amount of hydrogen-ions as determined by the concentration cell remains constant. Different indicators are affected in different directions. With some, the addition of salt induced an apparently more alkaline reaction, *e.g.* litmus, with others a more acid reaction, *e.g.* methyl-orange and alizarine. The latter are the two indicators used by Pauli and Handovsky and Hardy in their experiments.

We have repeated the experiments with the protein materials employed by these observers, using methyl-orange as an indicator, and obtained the same results as they did, that is to say, the change of colour invariably indicated *increase in acidity*.

However, in every case where direct measurement of hydrogen-ions was made, the solutions were found to be made more alkaline by the addition of sodium chloride; this was true of experiments made over a wide range of acidity, viz. from $10^{-1.7}$ normal to $10^{-4.08}$ normal, or 189000×10^{-7} normal to 832×10^{-7} normal, roughly between N/100 and N/10,000. These results are set forth in Table X.

Having ascertained that the addition of neutral salts to a slightly acid solution of protein produced a quite considerable diminution in acidity, it occurred to us that the fall in reaction-rate produced by salts might not be a direct effect of salts *per se* but be indirectly brought about through the diminution in hydrogen-ion concentration occasioned by their presence. We were somewhat strengthened in this opinion by the fact that we had previously discovered that within certain limits there exists a similar logarithmic relation, but in the inverse sense, between acid added to a protein solution and velocity of coagulation. If the logarithms of the average velocity in three experiments detailed in our previous paper (a) Table VI, see also Table XI below), made with a protein solution after addition of different amounts of acid per gram protein, be plotted against acid added, they fall upon a line, indicating

that as the acid varies arithmetically the velocity of reaction varies geometrically.

From a comparison of Tables IX and XI, however, it is seen that the diminution in acidity caused by the addition of NaCl is insufficient to account for the whole effect of the salt in lessening coagulation velocity. We were able to arrive at this conclusion because in the experiments set forth in Table XI a decrease in hydrogen-ion concentration from $10^{-5.30}$ × normal to $10^{-5.75}$ × normal, caused by addition of alkali, the coagulation-rate (average velocity) was reduced to $\frac{2}{3}$ths. In one of our experiments with salt (Table IX) an almost precisely

TABLE X. *Effect of addition of* NaCl *to a concentration of* 1 % *upon the reaction of solutions containing various proteins, previously acidified with various acids.*

Material	Total volume, c.c.	Acid added	Amount of acid added, in c.c. N/10 (or equiv.)	NaCl present %	Concentration of H^+ in terms of normality
Egg-white, diluted 1 in 32, boiled; 5 c.c. diluted to 8 c.c. by addition of water, acid, etc.	8	0	0	0	$10^{-8.00}$ normal ($0{\cdot}1 \times 10^{-7}$ N)
	8	$C_2H_4O_2$	0·7	0	$10^{-4.08}$ normal (832×10^{-7} N)
	8	,,	0·7	1·2	$10^{-4.25}$ normal (563×10^{-7} N)
	8	H_2SO_4	0·7	0	$10^{-2.89}$ normal (12900×10^{-7} N)
	8	,,	0·7	1·2	$10^{-3.64}$ normal (2300×10^{-7} N)
Horse-serum, dialysed, diluted 1 in 25; 25 c.c. diluted to 50 c.c. by addition of water, etc.	50	HCl	12	0	$10^{-1.75}$ normal (189000×10^{-7} N)
	50	,,	12	1·0.	$10^{-1.86}$ normal (178000×10^{-7} N)

TABLE XI. *Effect of change of acidity* (*induced by addition of different amounts of alkali to an originally acid solution*) *upon average coagulation velocity of* 1 % *egg-albumen solution at* 69° C. (*from previous paper, loc. cit., Table VI*).

No. of c.c. N/10 AmOH added per gm. protein	H^+ concentration, in terms of normality (from Fig. 7, *loc. cit.*)	Av. velocity of coagulation, during the period in which concentration of residual albumen was being reduced from 6 mgs. per c.c. to 3 mgs. per c.c., in mgs. per c.c.
0	$10^{-4.6}$ normal (251×10^{-7} N)	·4225
1·6	$10^{-5.3}$,, (50·1 ,,)	·0522
2·4	$10^{-5.75}$,, (17·8 ,,)	·0200

similar reduction in acidity (hydrogen-ion concentration reduced from $10^{-5\cdot30}$ × normal to $10^{-5\cdot73}$ × normal) was caused by increasing the concentration of sodium chloride from normal to twice normal; in this instance, however, the velocity constant was reduced to nearly $\frac{1}{14}$th of its value.

It follows, therefore, that a retardation of reaction by neutral salts is produced in two ways:

(1) To a small extent by lowering the hydrogen-ion concentration of the acid protein solution.

(2) To a large extent by some direct influence of strong salt solutions.

This effect is cumulative. If a particular addition of salt slows the rate by $\frac{1}{x}$, by a further addition of an equal amount the rate is reduced by $\frac{1}{x^2}$.

The tendency for the alteration of coagulation-rate of protein on addition of salt or acid to assume a logarithmic relationship to the salt or acid added is rather striking. It has been suggested to us by Mr W. B. Hardy that, assuming rate of reaction to be a function of total surface of particles of protein, such an effect would be produced if additions of salt and acid were to consistently increase and diminish respectively the mean size of the molecular aggregates, whereby the total reacting surface of the protein would be diminished logarithmically in the case of salt, and increased logarithmically in the case of acid. A similar explanation is suggested to explain the greatly enhanced effect of temperature upon the coagulation velocity of proteins (see below).

Effect of the presence of Am_2SO_4 *on the temperature coefficient of the reaction between egg-albumen and hot water.*

In our previous paper we showed that the temperature coefficient of the reaction called heat coagulation was, both in the case of hæmoglobin and egg-albumen, an extraordinarily high one, viz. 1·3 times per 1° C. with the former and 1·91 per 1° C. with the latter. In the presence of a concentration of Am_2SO_4 equal to twice normal the effect of temperature upon rate of reaction of egg-albumen appears from a comparison of the two experiments detailed in Table XII to be considerably less, viz. 1·57 per 1° C. At present it is not clear what the significance of these high temperature coefficients is, or how the presence of salt modifies them. It has been suggested that the phenomenon may be related to some

change in the state of aggregation of the particles with rise of temperature, whereby the extent of the reacting surfaces is greatly increased, in addition to the usual increase in molecule energy.

TABLE XII. *Effect of temperature upon coagulation velocity of egg-albumen in* 1 % *solution in presence of a concentration of* Am_2SO_4 *equal to twice* (2·03 ×) *normal.*

Temperature, °C.	Time, minutes, $=t$	Amount of filtrate analysed, c.c.	Weight of coagulum, grms.	Concentration of residual albumen, mgrs. per c.c. $=C$	$\frac{1}{C}$	$\frac{1}{t_n - t}\left(\frac{1}{C_n} - \frac{1}{C}\right)$
75·45	10	10	·0907	9·070	·110	·00114
	15	15	·1292	8·613	·116	·00114
	23	16·7	·1351	8·091	·124	·00115
	36	21·8	·1536	7·046	·142	·00112
	50	31·5	·1959	6·220	·161	·00108
	$130 = t_n$	23	·0932	$4·052 = C_n$	·247	—
					Mean	·00113
70·9	(From Table VIII)		...	...	...	·000148

It is necessary to again emphasise the fact that complete "heat coagulation" of proteins (as investigated by the method of estimating residual protein at successive intervals of time) consists of two processes:

(1) the union of the protein and hot water ("denaturation"),

(2) subsequent agglutination and separation of the product.

The first may take place without the second, as when a 10 % solution of serum or egg-white is boiled without adjusting the reaction. We have lately made a separate study of the agglutination process, the results of which will form another communication. We are convinced that in the above and in our previous experiments we have been investigating the nature of the first process, uncomplicated by the second, for the following reasons:

(1) The samples as they were taken from the partly coagulated solutions filtered easily and clearly, and the filtrates showed no further change on standing.

(2) The temperature coefficient of this first process in coagulation we have found to be extremely high, coagulation-rate being increased 1·3 and 1·9 times for rise of 1° C. in temperature in the case of hæmoglobin and egg-albumen respectively, whereas we have ascertained that the temperature coefficient of the second process—agglutination—under

circumstances in which this phenomenon is complete, is very much lower: about 2 to 2·5 for 10° C. rise in temperature or 1·10 for 1° C.

(3) The effect of salt supports the above conclusion, for whereas we find that the addition of sodium chloride and other salts facilitates the separation of the "denaturated" egg-albumen in a particulate form, it has been shown above to greatly delay the rate of coagulation as studied by us.

We therefore conclude that agglutination-rate is here much in excess of denaturation-rate, and that the velocity of the latter is the limiting factor of the complete process in all our experiments.

Summary and Conclusions.

1. "Heat coagulation" of egg-albumen consists of two processes, viz.

(1) the reaction between the protein and hot water ("denaturation"), and

(2) the separation of the altered protein in a particulate form (agglutination).

In the experiments which form the basis of the present and previous communications, it was always arranged that the rate at which (2) occurred was greatly in excess of that of (1). Accordingly, in our study of "heat coagulation" and the influence of various factors upon the rate of the reaction, we are merely concerned with (1), as this is the limiting factor in the process.

2. The action of hot water on egg-albumen, which constitutes the first stage in "heat coagulation," is, if means be taken to prevent change in acidity during the process, a reaction of the first order. The same was previously found to be true for hæmoglobin.

3. During the process, as the protein is precipitated, free acid is progressively removed from the solution. The consequent progressive diminution in the acidity of the solution accounts for the fact that the reaction appeared to be of a more complicated character.

4. Further experiments on the fixation of acid by pure egg-albumen in the cold, showing the relation of amount of acid fixed to the acidity of the solution and the reversibility of the process are presented.

5. The extra amount of acid fixed by egg-albumen on coagulation and its dependence (1) upon the total concentration of acid and (2) upon acidity (hydrogen-ion concentration) has been determined.

6. The disappearance of the small additional amount of acid on coagulation is explained as follows: coagulation disturbs the equilibrium between hydrolysed and unhydrolysed protein-salt since the unhydrolysed salt is acted upon by hot water and precipitated much more rapidly than the protein itself. This disturbance of equilibrium is adjusted by a corresponding diminution in the concentration of the products of hydrolysis, *i.e.* a combination of the protein with free acid. In this way the free acid progressively disappears.

7. The effect on coagulation-rate of the presence of salts (NaCl and Am_2SO_4) up to a concentration of three times normal has been studied and shown to greatly lower the rate of reaction.

8. Up to a concentration of salt equal to twice normal the effect on coagulation-rate varies geometrically as the additions of salt are varied arithmetically.

9. The addition of neutral salts to an acid protein solution disturbs the equilibrium between protein and acid so that less free acid is present. The influence of salts in lowering coagulation-rate may be to some extent thus explained, but the major part of the effect must be due to some direct influence of the salts upon the system.

REFERENCES.

(1) Chick and Martin. This Journal, XL. p. 404. 1910.
(2) Sutherland. This Journal, XLII. 1911. (Proc. Physiol. Soc. p. vii.)
(3) Hopkins and Pinkus. This Journal, XXIII. p. 130. 1898.
(4) Sörensen. Biochem. Ztschr. XXI. p. 131. 1909.
(5) Platner. Ztschr. f. Biol. II. p. 417. 1866.
(6) Robertson, T. Brailsford. Ergebn. d. Physiol. x. Jhrg. p. 216. 1910.
(7) Bugarszky and Liebermann. Pflüger's Archiv, LXXII. p. 51. 1898.
(8) Moore and Bigland. Biochem. Journ. v. p. 32. 1910.
(9) Sörensen and Jürgensen. Biochem. Ztschr. XXXI. p. 397. 1911.
(10) Michaelis and Rona. Biochem. Ztschr. XVIII. p. 317. 1909.
(11) Starke, K. V. Abstr. Maly's Jahresb. ü. Tierchem. XI. p. 17. 1881.
(12) Haycraft and Duggan. Journ. of Anat. and Phys. XXIV. p. 288, also Brit. Med. Journ. p. 167. 1890.
(13) Osborne and Campbell. Connecticut Experimental Station Report, p. 348, also Journ. Amer. Chem. Soc. XXIV. p. 422. 1900.
(14) Starke, Joh. Ztschr. f. Biol. XLII. p. 187. 1901.
(15) Pauli and Handovsky. Beitr. chem. Phys. u. Path. XI. p. 415. 1908.
(16) Devoto. Ztschr. f. physiol. Chem. XV. p. 465. 1891.
(17) Hopkins. This Journal, XXV. p. 306. 1900.
(18) Pauli and Handovsky. Biochem. Ztschr. XVIII. p. 340. 1909.
(19) Hardy. This Journal, XXXIII. p. 251. 1905–6.
(20) Michaelis and Rona. Biochem. Ztschr. XXIII. p. 61. 1909.

No. 3

THE BACTERICIDAL ACTION OF THE CRESOLS AND ALLIED BODIES AND THE BEST MEANS OF EMPLOYING THEM.

The investigation described in the following report was undertaken at the request of the Therapeutic Committee of the British Medical Association.

In this investigation the germicidal powers of the various members of the phenolic class of substances have been compared and the effect upon germicidal power of the introduction of different chemical groupings into their molecules have been studied.

As many of these phenols are very feebly soluble in water, it is necessary to use them in the emulsified form. Accordingly a comparison of the efficiencies of the various emulsificants has been made.

The value of the emulsified disinfectants as germicides has been determined when diluted with water and also under a condition frequently met with in practice, namely, the presence of particulate organic matter which is known to have such a detrimental effect upon their germicidal efficiencies. Certain other questions of importance in practice have also been considered such as the toxicity, irritating properties and alkalinity of the disinfectants, and also the cost of their preparation in relation to their germicidal powers.

I.—The Experimental Methods Employed for the Determination of Germicidal Power,

The principle of these methods is that of Rideal and Walker's (1903, Journ. of the Roy. San. Inst., Vol. XXIV., p. 424) and consists in the comparison of the concentration of the disinfectant under examination with the concentration of pure phenol in water required to kill certain micro-organisms in a certain time under standard conditions defined below. The ratio of these two concentrations is called the carbolic acid co-efficient, and this is taken as the measure of germicidal efficiency.

Method (i.) was the modification of the Rideal-Walker method, introduced by Chick and Martin (*Journal of Hygiene*, Vol. VIII., No. 5, November, 1908), the essential modification being the introduction of an arbitrary time during which the disinfectant was allowed to act. In this

test germicidal efficiency was determined in the absence of organic matter.

The culture-medium used was a mixture consisting, per litre, of Brand's Meat Juice 10 c.c., salt 5 gms., peptone 10 gms., glucose 10 gms. The mixture was heated and filtered, and the acidity of the filtrate determined by titrating a portion with standard sodium hydroxide. The medium had a standard acidity of +6 to +7, according to Eyre's notation.

Litmus was then added until the medium had a sufficiently blue tint. The addition of litmus facilitated the detection of growths of the organisms used, which formed acid from the glucose. The culture medium was sterilised by steaming on three successive days.

The germicidal powers of the various preparations were determined upon 24-hour cultures of B. typhosus and Staphylococcus pyogenes aureus grown at 37° C. and obtained by inoculating 6 c.c. of broth with a standard loopful of material removed from a stock agar culture which was grown at room-temperature (16°-20° C.).

In the actual determination of germicidal power the following experimental conditions were kept constant :—

(*a*) The temperature of disinfection=20° C.

(*b*) The time during which the disinfectants were permitted to act=15 minutes.

(*c*) The volume of the disinfecting fluid=5 c.c.

(*d*) The number of organisms introduced=5 drops of a 24-hours culture from a standard pipette delivering a drop ·02 c.c. in volume.

All apparatus—test-tubes, pipettes and flasks—and the distilled water used for diluting the disinfectants being previously sterilised, a series of test-tubes, some containing 5 c.c. of various concentrations of pure phenol and others the same volume of various concentrations of the disinfectants under examination, was placed in a water bath at 20° C. The tubes having taken the temperature of the bath they were inoculated at one-minute intervals with 5 drops of the culture from the standard pipette and well shaken. When fifteen minutes had elapsed since the first tube had been inoculated, by means of a platinum loop of standard size (holding a drop of water ·004 gms. in weight) two loopfuls of the disinfecting fluid were introduced into each of two glucose broth tubes containing 10 c.c. of broth. This process of sub-culturing was repeated at intervals of one minute from each of the remaining dilution tubes in succession, so that samples were removed in each case after the disinfectants in varying concentration had acted upon the germs for exactly fifteen minutes.

The sub-culture tubes were incubated at 37° C. and kept under observation for four days. The results of the incubation (the presence or absence of growth) were referred to the corresponding dilution tubes and indicated that certain concentrations of the disinfectants had killed the germs in fifteen minutes while others had not.

The carbolic acid co-efficient was obtained by dividing the mean of the smallest concentration of the disinfectant that killed the organisms in fifteen minutes and the largest that failed to do so into the mean of the corresponding concentrations of pure phenol.

Method (ii.) was similar to (i.) in technique, except that

particulate organic matter in the form of a 3 per cent.* suspension of finely powdered dried fæecs was introduced into the dilution tubes.

The particulate matter consists largely of bacteria, cellulose, fat, and other bodies extractable with ether. When fæces are introduced into a disinfectant fluid the tar acids are absorbed by the particles of organic matter, so that a considerable portion is put out of action.

As already stated, the standard time chosen during which the disinfectants were allowed to act was fifteen minutes. In practice, however, it may be of importance to know the germicidal power of a disinfectant when permitted to act in a shorter time such as two minutes.

In the following experiments the effect of time upon the germicidal efficiency of phenol and two coal-tar disinfectants—T and Y, in Table XII. containing respectively tar-acid fractions No. 4 and No. 10 emulsified with castor-oil soap—has been studied. Comparisons were made after 2 minutes' and after 15 minutes' disinfection, using B. typhosus and Staphylococcus pyogenes aureus. In all other respects the tests were carried out in accordance with the standard method described above.

The following results were obtained :—

Table I.

Disinfectant.	Organism.	Concentration required to kill in 2 min.	Concentration required to kill in 15 min.	Carbolic-acid co-efficients. After 2 min.	After 15 min.
Phenol	B. typhosus	13 in 1000	9·5 in 1000	—	—
Disinfectant Y	,,	·85 in 1000	·75 in 1009	15·3	12·6
Phenol	Staphylococcus	13 in 1000	8.0 in 1000	—	—
Disinfectant T	,,	4.0 in 1000	2.7 in 1000	3·2	2·9

The effect of altering the time from 2 to 15 minutes upon the germicidal efficiency of these particular disinfectants was therefore not very great.

Madsen and Nyman ("Zur theorie der Desinfection" Zeitschr. f. Hygiene, LVII., p. 388, 1907 and H. Chick, Journal of Hygiene, Vol. VIII., No. 1, Jan., 1908) showed that the velocity of disinfection increased greatly with rise in temperature. This is of importance in practice, since it shows that by employing a disinfectant in the warm a higher germicidal efficiency is obtained, and it has been taken into account in recommending the concentrations of the various disinfectants to be used for different purposes.

II.—The Germicidal Powers of the Cresols, Thymol, and the Halogen Derivatives of the Phenols.

The phenols are obtained from the distillates of coal-tar, namely, carbolic and creosote oil. The fraction distilling

* 3 per cent fæces was chosen as representing the amount of solid matter present if a stool containing 10 per cent. total solids were mixed with twice its volume of disinfectant.

from tar between 170°—230° C. is called carbolic oil and consists chiefly of carbolic acid and naphthalene, but it also contains cresols and some higher homologues of phenol. The phenols are extracted from the mixture by means of caustic soda; the separated alkali solution is decomposed with acid, and crude carbolic acid separates as an oil. Crude carbolic acid contains besides carbolic acid cresols and higher phenols. Pure carbolic acid can be separated by distillation, and the residue, also called crude carbolic acid, containing little phenol, but consisting chiefly of cresols, has been used as a disinfectant. Its germicidal properties are dealt with in this chapter.

The tar fraction distilling between 230°—270° C. is called creosote oil and contains carbolic acid, cresols, the higher phenols and hydrocarbons, such as naphthalene and anthracene. The phenols are separated from the hydrocarbons by extraction with alkali; the alkali extract is decomposed with acid and the mixture of phenols that separates as an oil is called crude mixture of tar acids. Its germicidal power in the emulsified state is dealt with in Chapter IV. By the fractional distillation of this mixture of tar acids the constituent phenols can be separated to some extent. Between 180°—182° C. carbolic acid distils over; the tar acids boiling between 190°—200° C. are chiefly cresols; and the various fractions boiling at higher temperatures contain the xylenols and other higher homologues of phenol. The germicidal powers of these fractions emulsified with soap are described in Chapter IV.

Thymol is also a homologue of phenol, namely, 3 methyl—6-isopropyl-phenol. It is a white solid obtained from Oil of Thyme by extraction with alkali and precipitation from the extract with acid.

A. *The solubilities of the phenols.*

A saturated solution of phenol in water at room temperatures contains 7½ per cent. of phenol; the three isomeric cresols do not form aqueous solutions above 1·25 per cent.; while thymol is so feebly soluble that its saturated solution is 1 in 1,500. The other homologues of phenol and cresol are also very feebly soluble in water. The cresols and higher tar acids are, therefore, usually employed in the form of fine emulsion produced by means of soaps, glue, and gum.

B. *The germicidal power of the cresols and of thymol in aqueous solution.*

Fränkel (1889, Zeitschr. f. Hygiene, Vol. VI., p. 521) showed that the superiority in germicidal power of crude carbolic acid over pure phenol was due to the presence of the homologues of phenol. He found by means of experiments with anthrax spores that the pure ortho, meta, and para cresols possessed germicidal power considerably greater than that of phenol.

Henle (1889, Archiv f. Hygiene, Vol. IX., p. 188) shewed that creolin contained cresols and their higher homologues and that both creolin and the cresols possessed a greater germicidal power than phenol upon B. typhosus and Staphylococcus pyogenes aureus. He found that the germicidal powers of the cresols varied with their boiling-points, meta-cresol with the highest boiling point being the most efficient and the ortho-compound with the lowest being the least efficient of the three isomers. He also showed that the phenols

isolated from creolin exceeded the cresols in germicidal power. This was due to an admixture of some high boiling-point phenols with the cresols in creolin.

Blyth and Goodban (1907, May, *Analyst*) have shown by experiments with B. coli that the three isomeric cresols emulsified with soap possessed practically the same germicidal power, which was 2·1 times as great as that of phenol also emulsified with soap.

Rapp (1909, Desinfektion, Heft. 12. 2. December) shewed that the presence of such substances as pyridin and anthracene associated with the cresols in coal-tar decreased the germicidal powers of the latter substances. He also shewed that the cresols were more effective germicides when emulsified with linseed oil soap and palmitic acid soap than when emulsified with the soaps of oleic and stearic acids. The addition of resin-soap he found increased the germicidal powers of saponified cresols.

Jalan de la Croix (Archiv f. exp. Path., January 20, 1881) found that on an average 1 in 1,000 thymol inhibited the growth of bacteria, but 1 per cent. solutions were necessary to kill them.

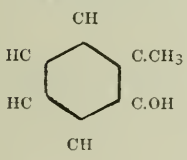
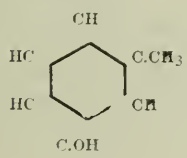
Ratimoff (Biedermann's Centralblatt, Vol. XIV., p. 360) considered thymol a strong germicide, and placed it fourth in his list of disinfectants arranged according to potency (mercuric chloride, silver nitrate, iodine, thymol). Kobert, Kohler, and Stern have used thymol to preserve vaccine lymph.

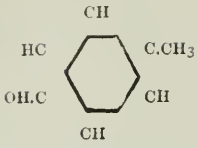
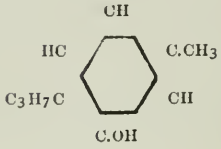
The germicidal powers of the cresols and thymol were first determined in aqueous solution with B. typhosus and Staphylococcus pyogenes aureus.

(I.) *In the absence of organic matter.*

The results are expressed as carbolic co-efficients in the following table :—

TABLE II.

Organism.	*Germicide.*	*Carbolic Co-efficient.*
B. typhosus.	Ortho-cresol. 	2·6
,,	Meta-cresol. 	2·6

Organism.	*Germicide.*	*Carbolic Co-efficient.*
B. typhosus.	Para-cresol.	
,,		2·6
	Thymol.	
,,		25·0
Staphylococcus pyogenes aureus.	Ortho-cresol	2·1
,,	Meta-cresol.	2·0
,,	Para-cresol.	2·4
,,	Pure commercial cresylic acid. (Admixture of the three cresols)	2·2

The cresols were therefore nearly equal in germicidal power, but were considerably more efficient than phenol.

Thymol, possessing an isopropyl as well as a methyl group in the aromatic nucleus, was twenty-five times as powerful a disinfectant as phenol and ten times as powerful as the cresols to B. typhosus.

These facts show that accumulation of alkyl groups in the benzene ring of phenol may increase germicidal power to an enormous degree. High germicidal power was therefore to be sought in the higher homologues of phenol. These are found commercially in the higher boiling-point fractions of the tar acids derived from creosote-oil.

The carbolic coefficients of the cresols were greater in the case of B. typhosus than in the case of Staphylococcus. This indicates that the difference in the resistances of these organisms to the action of germicides was more marked in the case of the cresols than with phenol.

(*ii.*) *In the presence of particulate organic matter* (*3 per cent. dried fæces*).

Organism.	*Disinfectant.*	*Carbolic-acid co-efficient.*
Staphylococcus py-aur.	Cresylic Acid.	1.7

In the absence of organic matter the same sample of cresylic acid possessed a coefficient of 2·2 (see above).

The presence of organic matter in the form of a 3 per cent. suspension of dried fæces somewhat decreased the bactericidal power of phenol. For, while 9 to 10 in 1,000 phenol killed Staphylococcus in 15 minutes in the absence of organic matter,

11 to 13 in 1,000 phenol was required to kill the same organism in the same time in the presence of the organic matter. The depressing effect of particulate organic matter upon the carbolic coefficient of cresylic acid therefore meant that the bactericidal action of cresylic acid was a little more decreased by the organic matter than was that of phenol.

C.—*The germicidal powers of the emulsified cresols and thymol.*

(I.) *a.* In absence of organic matter. *b.* In presence of 3 per cent of dried fæces.

(i.) *The advantages of employing the cresols in the suspended state.*—(*a*) As has already been pointed out, the limited solubility of the cresols in water prevents their aqueous solutions from always being serviceable as disinfectant fluids. Stronger solutions in water than 1·25 per cent. at ordinary temperatures cannot be obtained, and often stronger solutions are required in practice. Recourse must therefore be had to the method of obtaining permanent suspensions of the cresols in other media. For this purpose a soap is very convenient and has for a long time been employed in the preparation of coal-tar disinfectants. By warming cresylic acid with soft soap until all the soap has dissolved, a dark-coloured fluid is obtained which is freely miscible with water with no separation of cresols, so that much stronger concentrations of cresols can be obtained in this way than by dissolving them in water alone.—(*b*) The same concentration of bactericidal substance may have a greater germicidal power when emulsified than when dissolved, as was shown by Chick and Martin [Journal of Hygiene, Vol. VIII. No. 5, November, 1908] and by Massey [Journal of Hygiene, Vol. IX., No. 3, November, 1909]. This advantage, however, only applies to higher concentrations of the cresol-soap, because in the case of concentrations below 1·25 per cent. that are used in investigating the bactericidal power of the cresols with the moderately resistant organisms—B typhosus and Staphylococcus—the cresol is completely in solution and is no longer suspended in the soap solution. In higher concentrations part of the cresol is emulsified and part is in solution and the bactericidal superiority of an emulsified disinfectant must come into operation. This advantage in using emulsified cresol is therefore not made evident when only moderately resistant organisms are used.

(*c*) That the soap present exerts a small bactericidal action and thus intensifies that of the cresol.

(*d*) That the method of suspension does not destroy the transparency of the diluted preparations.

(ii.) *The best methods of preparing cresol emulsions.*—(*a*) The first method that gave good results was that employed for making up the Liquor cresoli saponatus of the German, Japanese, and the Swedish Pharmacopœias, and the same preparation, official in Belgium and Switzerland, called Cresolum saponatum. Equal weights of the cresols and B.P. soft soap were warmed together until all the soap had dissolved. A reddish-brown fluid resulted which was completely miscible with water, yielding clear dilutions.

Table III.

Nature of Cresol-body emulsified with Soft-soap.	Germicidal Efficiency.				Concentrations * recommended for use in practice.					
	Staphylococcus Pyogenes Aureus.		B. typhosus.		For washing hands or sterilising instruments (absence of organic matter).		For the disinfection of *typhoid stools*.		For the disinfection of *pus from empyemas*.	
	Carbolic acid co-efficient, in absence of faeces.	Carbolic acid co-efficient, in presence 3 per cent. faeces.	Carbolic acid co-efficient, in absence of faeces.	Carbolic acid co-efficient, in presence of faeces.			(The concentrations given below are to be added to ½ their volume of stools or pus.)			
					At 20°C.	At 35°C.	At 20°C.	At 35°C.	At 20°C.	At 35°C.
A. Pure commercial Cresylic Acid	1·2	1·3	—	—	2%	1%	3%	1½%	—	—
B. Tar-acid, Fraction No. I., 193°—199·5° C.	1·4	1·2	—	—	2%	1%	3%	1½%	—	—
C. Tar-acid, Fraction No. II, 195°—202·5° C.	1·6	1·2	3·0	1·8	2%	1%	3%	1½%	—	—
D. Tar-acid, Fraction No. III., 202·5°—205° C.	1·4	1·3	—	—	2%	1%	3%	1½%	—	—
F. Crude carbolic acid....	·9	·9	1·5	1·1	2%	1%	3%	1½%	3%	1½%

* The concentrations recommended for practical purposes are based on the experimental results and by means of the preliminary experiments with two-minutes germicidal action are adapted for short periods of disinfection (say 2 minutes). An additional allowance has also been made for the fact that organisms that have been living recently in the animal body and therefore at a high temperature are somewhat more resistant to disinfectants than the organisms of a laboratory culture that have not been recently passaged.

Preparations were made according to this method of pure commercial cresylic acid, of the fractions of tar acids from creosote-oil, that came over in the neighbourhood of the boiling-points of the cresols (Fractions I., II., III., 193°-205° C.), and also of crude carbolic acid.

The bactericidal powers of these five preparations upon Staphylococcus pyogenes aureus and B. typhosus were determined in the presence and absence of fæces. The results are set forth in the table on page 8.

The fractions boiling at the temperatures indicated in table III. contained the cresols as the principal constituents. They all yielded when warmed with an equal weight of soft soap a reddish-brown homogeneous fluid completely miscible with water and giving clear dilutions with distilled water, but slightly turbid ones with tap-water, owing to the precipitation of some of the soap by calcium and magnesium salts. The preparations made from pure cresylic acid and these fractions possessed practically the same carbolic coefficient. Their bactericidal power was not influenced by the presence of a 3 per cent. suspension of fæces to a much greater extent than that of phenol.

The germicidal efficiency of the Liquor cresoli saponatus prepared with crude carbolic acid was considerably less than that of the same prepared with the tar-acid distillates consisting almost entirely of cresols and boiling from 193°-205° C.

(*b.*) Another cresol suspension that was worked with was the Liquor cresolis compositus of the United States Pharmacopœia. The formula of this preparation is thus: Cresylic Acid, 50; Linseed Oil, 35; Potassium Hydroxide, 8; Water to make up to 100 by weight. This mixture was gently warmed and a brown fluid resulted. Complete saponification of the fat, however, was not effected, so that on dilution there was a great separation of fat.

The carbolic co-efficient of this preparation determined with Staphylococcus pyogenes aureus and in the absence of organic matter was ·8. Its bactericidal power was therefore less than that of carbolic acid. The carbolic acid coefficient of the cresylic acid used in making this preparation was 2·2 in aqueous solution with Staphylococcus and in absence of organic matter, so that, since the preparation contained 50 per cent. cresylic acid, its carbolic acid coefficient should have been a little over 1·1, as the soap would exert a small bactericidal action.

There was quite a measurable difference between ·8 and 1·1 and the reason for this falling-short in bactericidal power was discovered in the fact that the preparation contained an excess of fat in consequence of incomplete saponification. When diluted with water a milky fluid was formed due to the presence of fat globules. The cresols are extremely soluble in fats so that the active substances were partitioned between water and fat and a considerable portion was thus put out of action.

(*c.*) The difficulty of effecting the complete saponification of linseed oil led to experiments with the next cheapest oil, castor oil. It was found that the bleached and filtered castor oil of commerce, costing 9d. per lb., was very readily saponifiabl in the presence of tar-acids.

Disinfectant fluids were first of all prepared with castor oil according to the formula above used in the case of linseed oil, but it was found practicable to alter the formula by reducing the proportion of oil and still obtain equally good emulsions. The formula finally adopted was the following :—

Cresols	50
Castor Oil.....	20
Caustic Potash	5
Water	5, all by weight.

Preparations were made with crude carbolic acid and the fraction III. of tar acids, boiling from 202·5°-205° C. The other two fractions (consisting almost entirely of cresols) boiling at 193°-199·5° C. and 199·5°-202·5° C. could, of course, be substituted for fraction III. if desired.

The castor oil and tar acid in the above proportions were well mixed and heated together in the water bath; the alkali was then dissolved in the water and the solution, being already hot owing to the heat of solution of the potash, was added to the hot mixture of tar acid and fat. Heating in the water bath was continued until the saponification of the fat was complete, this stage being reached when there was no longer a fatty scum produced when a drop of the mixture was diluted with water.

The products were dark-coloured, translucent, homogeneous fluids that gave beautifully clear dilutions with distilled water and somewhat turbid ones with tap-water.

The modification in the formulæ of these preparations, consisting in the reduction of the proportion of fat, had the following advantages :—

(i.) The final preparations possessed a higher tar-acid content and a consequent higher germicidal efficiency.

(ii.) The dilutions with tap-water were much less turbid than those of the preparations containing the excess of castor-oil soap.

(iii.) The cost of production of the preparations was less.

The results of the determination of their germicidal powers are given in Table IV. on page 11.

From these results the following conclusions can be made :

1. That the germicidal powers of the cresol castor-oil soap preparations were not severely depreciated by the presence of 3 per cent. suspensions of faeces.

2. That the preparations made with crude carbolic acid were less efficient than those containing the cresol fractions.

3. That on comparing the germicidal efficiencies of the castor-oil soap preparations with those of the corresponding soft-soap preparations (Table III.) it is found that the former are the superior germicides owing to their higher content of tar acid (62½ per cent. as against 50 per cent. tar acids).

Table IV.

Nature of Cresol. Containing tar-acid and formula of preparation.	Germicidal Efficiency.				Concentrations recommended to be used in practice.			
	Staphylococcus py. aur.		B. typhosus.		For washing hands and sterilising instruments.		For disinfection of typhoid or other stools. (Concentration to be added to half volume of stools.)	
	Carbolic-acid co-efficient in the absence of fæces	Carbolic-acid co-efficient in the presence of 3 per cent. fæces	Carbolic-acid co-efficient in the absence of fæces.	Carbolic-acid co-efficient in the presence of 3 per cent. fæces.	At 20° C	At 35° C	At 20° C	At 35° C
H Crude Carbolic Acid .. Tar Acid, 50 by wt. .. Castor Oil, 20 „ .. Potash, 5 „ .. Water 5 „ ..	1·6	1·7	3·6	2·2	2%	1%	3%	1½%
I Tar Acid Distillate .. Fraction No. III. .. 202·5°—205°C .. .. Tar Acid, 50 by wt. .. Castor Oil, 20 „ .. Potash, 5 „ .. Water, 5 „ ..	2·9	1·9	5·2	3·4	1%	½%	2%	1%

Table V.

Disinfectant.	Germicidal Power.				Concentrations recommended for use in practice.			
	Staphylococcus.		B. Typhosus.		For washing hands and sterilising instruments, absence of organic matter.		For disinfection of typhoid stools. Concentrations to be added to ½ their volume of stools.	
	Carbolic-acid co-efficient in the absence of fæces.	Carbolic-acid co-efficient in in the presence of fæces.	Carbolic-acid co-efficient in the absence of fæces.	Carbolic-acid co-efficient in the presence of fæces.	At 20°C.	At 35°C.	At 20°C	At 35°C.
J Crude Carbolic-acid emulsified with Resin	1·9	1·4	2·5	1·8	1½%	¾%	2½%	1¼%
K Tar-acid Fraction No. III. [202·5° to 205° C.] emulsified with Resin	3·0	1·7	3·6	2·6	1%	½%	2½%	1¼%

(D) *The use of alkali-resin as emulsificant.*

The resin used was common amber. The formula for the preparation was the following :—

Tar-acid	...	50
Resin	...	25
Potash	...	6
Water	...	5 by weight.

The tar-acid and resin were heated together on the water-bath until all the resin had dissolved. The potash dissolved in the water was then added and the mixture well stirred and heated until a drop of the mixture gave a milky dilution with water with no separation of tar-acid. Disinfectant-fluids were prepared according to this formula with crude carbolic-acid and one of the cresol-fractions (No. III.) of the tar-acids. They were dark-coloured, homogeneous, somewhat viscous fluids which gave milky dilutions with both distilled and tap-water, in this differing from the corresponding preparations made with soft-soap and castor-oil soap.

The bactericidal powers of these preparations were determined and the experimental results are given in Table V. on page 12.

From these results the following conclusions may be drawn :

(i.) The presence of a 3% suspension of fæces depreciated to a certain extent the germicidal value of the cresols emulsified with resin soap.

(ii.) The germicidal power of the preparation containing crude carbolic-acid was less than that of the preparation containing one of the cresol-fractions of the tar-acids.

(iii.) As indicated in the comparative table (page 12), the cresols preparations emulsified with resin soap and containing 58% of tar-acid were generally a little weaker in germicidal power than the castor oil soap preparations containing 62·5% of tar-acid :—

TABLE VI.

DISINFECTANT.	CARBOLIC ACID CO-EFFICIENT.		
	Staphylococcus py. aur.	B. Typh. In absence of fæces.	B. Typh. In presence of fæces.
(J) Crude carbolic acid, emulsified with resin	1·9	2·5	1·8
(H) Crude carbolic acid, emulsified with castor-oil soap	1·6	3·6	2·2
(K) Tar-acid, Fraction III., emulsified with resin	3·0	3·6	2·6
(I) Tar-acid, Fraction III., emulsified with castor-oil soap	2·9	5·2	3·4

(iv.) A comparison of the germicidal powers of the cresols emulsified with resin soaps with those of the cresols emulsified with soft soap (Table III.) shows that the resin-preparations were the superior germicides owing to the higher content of tar-acid.

This is indicated in the following comparative table :—

TABLE VII.

DISINFECTANT.	CARBOLIC CO-EFFICIENTS.			
	Staphylococcus.		B. Typhosus.	
	In the absence of fæces.	In the presence of fæces.	In the absence of fæces.	In the presence of fæces.
Crude carbolic acid, emulsified with an equal amount of soft soap.	·9	·9	1·5	1·1
Crude carbolic acid, emulsified with resin according to this formula.— Crude carbolic acid .. 50 Resin .. .. .. 25 Potash .. .. .. 6 Water .. .. .. 5 All by weight.	1·9	1·4	2·5	1·8
Tar-acid Fraction III. (cresols), emulsified with an equal amount of soft soap.	1·4	1·3	—	—
Tar-acid Fraction III. (cresols), emulsified with resin according to this formula:— Tar-acid .. .. 50 Resin .. .. .. 25 Potash .. .. .. 6 Water .. .. .. 5 All by weight.	3·0	1·7	3·6	2·6

(iii.) *A comparison of the germicidal properties of cresol in aqueous solutions and in soap-suspension.*

The results of the comparative experiments are set forth below :—

TABLE VIII.

Nature of Cresol and and medium of solution or suspension.	Organism.	Carbolic-acid co-efficient of *Cresols.*	
		In absence of fæces.	In presence of fæces.
Pure commercial Cresylic Acid examined in aqueous solution	Staphylococcus.	2·2	1·7
The same examined in soap-suspension (containing 50% of tar-acid suspended with 50% soft-soap.....	,,	2·5	2·6

By suspending the cresols with soap there results a slight increase in their germicidal power. This is not to be attributed to the condition of emulsification of the cresols in the soap because at the concentrations (5 in 1000) used in determining the carbolic-acid co-efficients the cresols would be in true solution. The increased germicidal power is probably due to the germicidal properties of the soft-soap present.

(iv) *The germicidal properties of emulsified thymol.*

The high germicidal power of thymol in aqueous solution has already been pointed out, but its limited solubility in water is a practical disadvantage.

Although thymol is official in the British Pharmacopæia, there is no official soap emulsion of it. Experiments were therefore carried out to produce such a preparation. The best method of emulsification consisted in heating 50 parts of thymol with 20 parts of castor oil and saponifying the latter with a solution of 5 parts of potash dissolved in five of water.

The product was a dark-coloured homogeneous fluid which gave a white stable emulsion of thymol with water.

The germicidal properties of this preparation were examined and the results are tabulated below :—

TABLE IX.

B. TYPHOSUS.		STAPHYLOCOCCUS PY. AUR.	
Carbolic co-efficient in absence of fæces.	Carbolic co-efficient in presence of 3 per cent. dried fæces.	Carbolic co-efficient in absence of fæces.	Carbolic co-efficient in presence of 3 per cent. dried fæces.
30	8·8	23	7·5

The emulsified thymol thus possessed a very high germicidal power which was greater than any of the emulsified tar-acid fractions dealt with in Chapter IV. Its efficiency, however, was greatly decreased by the presence of a 3% suspension of dried fæces.

(ii.) *The effect of the presence of tap-water on the germicidal powers of the preparations of the cresols.*

The determination of germicidal power was carried out in the usual way except that the dilutions of the disinfectants and of the phenol were made with sterile tap-water. Comparative experiments with distilled water using the same culture were also set up.

The experiments were carried out with a disinfectant in which tar-acids fraction No. 1 (consisting of cresols) was suspended with castor-oil soap in the following proportions :—

Tar-acid	50
Castor-oil	35
Potash	8
Water	7 all by weight.

The organism used was Staphylococcus pyogenes aureus. The results are set forth below :—

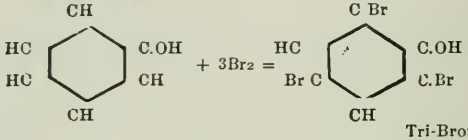
TABLE X.

DISINFECTANT.	DISTILLED WATER.		TAP WATER.	
	Carbolic-acid co-efficient.	Concentration killing in 15 mins.	Carbolic-acid co-efficient.	Concentration killing in 15 mins.
Phenol	—	11 in 1000	—	12 in 1000
Disinfectant G ..	2·3	4 6 in 1000	2·3	5·2 in 1000

Tap-water had, in these experiments, no effect upon the germicidal efficiency of either phenol or saponified cresol, although it precipitated some of the soap of the latter.

(D.) *The germicidal powers of the halogen derivatives of the phenols.*

Chlorine and bromine combine directly with phenols, forming halogen substitution products in which the halogen has replaced some of the hydrogen atoms of the benzene nucleus. This is shewn in the following equation :—

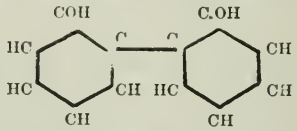


Tri-Brom-Phenol.

Mono-, di-, tetra- and penta- substitution products also exist.

Although these compounds are very insoluble in water, Bechhold and Ehrlich (1906. Zeitschr. f. Physiol. Chem. Hoppe-Seyler 47, p. 173) have determined their inhibitory powers upon the growth of B. diphtheria. They found that inhibitory power increased as the number of halogen atoms introduced into the benzene ring became greater. Thus as an inhibitor penta-brom-phenol was 100 times as efficient as phenol.

They also showed that halogen derivatives of ortho-bi-phenol



possessed great inhibiting power. Thus the tetra-brom-derivative was 500 times as efficient as phenol.

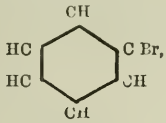
O-biphenol and its isomers are contained in some proprietary disinfectants.

Some of the halogen derivatives of phenol and cresol dissolved in glycerin or sodium hydroxide have been employed for disinfecting purposes, but these are not ideal media.

Attempts have been made to emulsify some of these halogen compounds, but the experiments up to the present have not been successful.

To obtain these substances in a form suitable for germicidal purposes is of importance because according to Bechhold and Ehrlich's work they are not extremely poisonous to the higher animals. For example, the fatal dose by subcutaneous injection for mice of tetra-brom-ortho-cresol is 0.44 gms. for 1000 gms. body weight.

Some experiments have also been carried out with a view to the emulsification of brom-benzene—



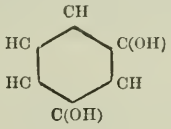
a liquid very insoluble in water. Unlike phenol and cresol, however, it does not dissolve soft-soap or castor-oil soap but forms with them yellow pastes of a non-homogeneous character. These pastes mix with water, forming white emulsions which are very unstable. No determinations of germicidal power have been made.

III.—The Bactericidal Powers of the Di-oxy and Tri-oxy Benzenes and of Quinone.

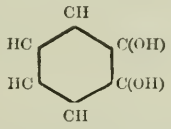
(i.) *The di-oxy-benzenes.*

There are three isomeric di-oxy-benzenes which may be regarded as derived from phenol by the replacement of a hydrogen atom in the benzene nucleus by the hydroxyl (O H) group. The constitutions of the substances are as follows :—

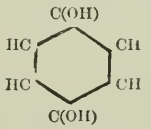
Resorcinol
(Meta-di-oxy-benzene)



Pyrocatechol
(Ortho-di-oxy-benzene)



Hydroquinone
(Para-di-oxy-benzene)



These substances are white solids which are readily soluble in water.

Their solubilities at 15°c are given below :—

(Saturated solutions) Resorcinol 4 parts in 3 parts of water
Pyrocatechol 1 part in 3.2 ,, ,, ,,
Hydroquinone 1 part in 17 ,, ,, ,,

Callias ("Ueber das Resorcin," Wurtzburg, 1880) showed that resorcinol possessed considerable restraining power upon the growth of micro-organisms. A 1 per cent. solution was able to preserve many animal fluids and a 2 per cent. solution prevented milk becoming sour.

Duggan (Amer. Chem. Jour., Vol. VII., p. 62) has compared the inhibiting powers of these di-oxy-benzenes by determining the amounts required to prevent the development of B. subtilis in beef-peptone.

His results are expressed in the following table, the inhibiting power of phenol being taken as the standard :—

Substance.	*Inhibiting co-efficient.*
Phenol	1
Pyrocatechol	1
Resorcinol	1·25
Hydroquinone	1·50

The inhibiting powers of phenol and pyrocatechol were thus equal and were somewhat less than the inhibiting power of resorcinol, which in turn was less than that of hydroquinone.

The germicidal powers of these substances have been determined with B. typhosus in absence of organic matter. Their carbolic co-efficients are tabulated below :—

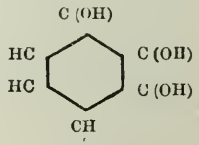
Substance	*Carbolic co-efficient.*
Resorcinol	·3
Pyrocatechol	·5
Hydroquinone	1·0

The introduction of a second hydroxyl group into the phenol molecule was thus accompanied by a considerable decrease in germicidal power, except in the case of hydroquinone the carbolic co-efficient of which was the same as that of phenol.

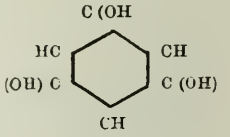
(ii.) *The Tri-oxy benzenes.*

There are three isomeric tri-oxy-benzenes, namely :—

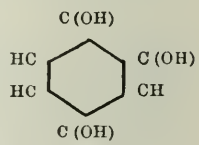
1.2.3. Tri-oxy-benzene, (Pyrogallol)



1.3.5. Tri-oxy-benzene, (Phloroglucin)



1.2.4. Tri-oxy-benzene,(Oxyhydroquinone)



These three compounds are very readily soluble in water.

Bovet (" Revue d'hygiene," 1879, p. 154) found that 2% solutions of pyrogallol inhibited the growth of bacteria and moulds while 3% killed bacteria. Duggan (l.c.) compared its inhibiting power with the inhibiting powers of phenol and dioxy-benzenes and found that pyrogallol was feebler than any of these compounds.

The germicidal powers of pyrogallol and phloroglucin were determined with B. typhosus, and their carbolic co-efficients are given below :—

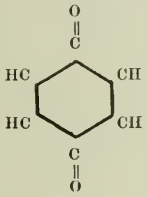
Substance.	*Carbolic co-efficients.*
Pyrogallol	·77
Phloroglucin	under ·35

These two tri-oxy-benzenes were therefore weaker than phenol in germicidal efficiency.

The results with the di-oxy and tri-oxy-benzenes lead us to a conclusion of practical importance, namely, that high germicidal power is not to be sought in phenols containing numerous hydroxyl groups.

(iii.) *The germicidal power of Quinone.*

Thalhimer and Palmer in the Journal of Infectious Diseases, Vol. IX., 1911, pp. 172-189, have shown that quinone, which has the following constitution and which



is an oxidation product of many benzene derivatives, considerably exceeded phenol and cresols in germicidal power. They found that with B. typhosus it had a carbolic co-efficient of about 84. Quinone forms addition-products with many phenols. Pheno-quinone—the addition compound of quinone and carbolic acid $C_6H_4O_2$, 2 C_6H_5OH—was also found to possess a high germicidal efficiency which was less, however, than that of quinone itself.

Some experiments have been made to compare the germicidal power of quinone with that of phenol and of an aliphatic ketone, such as acetone, $CH_3.CO.CH_3$, using Staphylococcus pyogenes aureus. The results are tabulated below :—

Substance.	*Carbolic co-efficient.*
Quinone	10
Acetone	Under ·075

The germicidal power of quinone upon Staphylococcus was therefore much greater than that of phenol, while the germicidal power of acetone was insignificant.

Quinone and many of its derivatives are not freely soluble in water, and it would, therefore, be of practical importance to prepare some fluid derivatives and emulsify them.

Quinone dissolves readily in crude carbolic acid, forming presumably a compound with the cresols. On warming soft-soap with this combination the soap dissolves, but the fluid product is decomposed by water with the formation of a black precipitate. Its germicidal power has not been determined.

IV. The Bactericidal Action of the Higher Tar-Acids.

A (*i.*) *In the absence of organic matter.*

(*ii.*) *In the presence of* 3 *per cent. dried fæces.*

The higher tar-acids used were various fractions of tar-acids derived from creosote-oil boiling from 205°-286°C. Preparations of these were made according to the formula used in the preparation of Liquor cresolis saponatus [50 per cent. Fraction; 50 per cent. soft-soap; the mixture being warmed until all the soap had dissolved]. Reddish-brown fluids were in this way obtained which were miscible with water giving milky fluids. The milkiness of these dilutions is due to the fact that the addition of water to the soap-suspensions of the higher tar-acids causes a separation of the tar-acids in a fine emulsion. A 1 per cent. dilution of the soap-suspension of Fraction IV. boiling between 205° and 209° C., however, was clear, but a 2·5 per cent. dilution was milky. This was probably due to the high cresol-content and low xylenol content of the preparation, the particles of insoluble tar-acid becoming so scarce in the 1 per cent. dilution that the milkiness disappeared.

The carbolic acid coefficients of these preparations were determined with Staphylococcus pyogenes aureus both in the absence and in the presence of particulate organic matter. It is seen that the carbolic-acid coefficients rise as the boiling points of the tar-acids rise. This fact is taken advantage of in various proprietary preparations. It is due not only to the fact that the bactericidal powers of the members of the phenol series increase with their molecular weights but also to the intensifying effect of emulsification upon bactericidal power. Chick and Martin (*Journal of Hygiene*, Vol. VIII., No. 5, Nov., 1908) showed that the increased efficiency of a germicide in the emulsified form was caused by an appropriation of the particles of the tar-acids by the bacteria. This appropriation was demonstrated by mixing a suspension of bacteria and an emulsion of tar-acids together and after allowing to stand for a short time centrifuging. The bacteria carried down a large amount of the tar-acids, while there was very little settlement when the tar-acid emulsion alone was centrifuged. By suitably arranging the proportions of tar-acids and bacteria the emulsion completely lost its opacity.

In consequence of this adsorption of the particles of tar-acids by the bacteria the latter must rapidly become surrounded by a much greater concentration of disinfectant than that which exists throughout the liquid, and by this local accumulation of bactericide the efficiency is greatly increased. Particulate organic matter also removes phenols and cresols from their aqueous solutions, but the extent of absorption is very much less than that which goes on in the case of emulsions, and this explains the superiority of emulsified disinfectants as bactericides. This effect of emulsification was not noticeable in the case of soap-suspensions of the lower tar-acids such as the cresols because at the dilutions used in determining bactericidal power the cresol had passed entirely into

aqueous solution. This insolubility of the higher tar-acids thus enables the bactericidal test to give a truer value for the disinfecting-power of their soap-suspensions.

We have seen that the presence of particulate organic matter (3 per cent. fæces suspension) had very little influence upon the bactericidal power of Liquor cresoli saponatus. With analogous preparations of the higher tar-acids this was not the case. The presence of the organic matter had a

TABLE XI.

Tar-acid body emulsified.	Carbolic-acid co-efficient.		Properties of dilutions.	
	In the absence of organic matter.	In the presence of organic matter. 3% fæces.	2·5%	1·0%
M Fraction IV. 205-209°C	1·7	1·4	Turbid. Dark-yellow.	Clear yellow.
N ,, V. 209-213°C	2·4	1·9	Turbid. Dark-yellow.	A little turbid. Yellow.
O ,, VI. 213-218·5°C	3.0	1·9	Turbid. Reddish-yellow	Somewhat turbid. Yellow.
P ,, VII. 218·5-225·5°C	3.8	2·0	Turbid. Reddish-yellow.	Somewhat turbid. Yellow.
Q ,, VIII. 225·5-240°C	4·5	2·0	Yellow and turbid.	Yellow and turbid.
R ,, IX. 240-247°C	8.7	2·4	Pink and milky.	Pink and milky.
S ,, X. 274-286°C	11 to 14	1·3	Pink and Milky Considerable immediate separation of emulsified tar-acid.	

marked depreciating effect upon the bactericidal powers of these preparations and this increased with the boiling-point of the fractions. The factor of emulsification had again come into operation. An emulsified disinfectant is much more greatly reduced in efficiency by particulate organic matter than a dissolved one; and, as in the dilutions of the cresol-preparations used in carrying out their standardisation the tar-acids were dissolved in water and in those of the higher tar-acid preparations they were still emulsified, the sensitiveness of the latter and the resistance of the former to particulate organic matter are both explicable.

This method of emulsification with soft soap not only afforded a means of obtaining some insight into the bactericidal action of the tar-acids, but was also practicable for the production of preparations containing the cresols (Crude Carbolic Acid and Tar-Acid Fractions I., II., and III.) which yielded clear solutions with water and which were superior to, or at least about equal to, carbolic acid in bactericidal power.

This method, however, was not serviceable for the production of preparations of the higher tar-acids suitable for practical purposes, since the emulsions of the tar-acids generated on diluting these preparations with water were not permanent, but in the course of a few hours the heavy tar-acids began to settle down forming an oily layer on the bottom of the vessel. Further, it was not found possible to emulsify even temporarily Fraction No. X. 276-284° C. with soft soap. On warming equal weights of this tar-acid fraction and soft soap a dark-coloured fluid was obtained, from which on dilution with water there was considerable separation of unemulsified tar-acid, which immediately settled down, and for this reason an accurate estimation of germicidal action of the preparation, the carbolic acid coefficient of which varied from 11 to 14, was not possible.

In the presence of lower fractions these tar-acids of higher boiling-point would no doubt form more stable emulsions.

A search was therefore made for more efficient emulsificants than soft soap, and after examining the emulsifying properties of various substances, which are described in Chapter V., castor-oil soap was chosen as the emulsificant for the higher tar-acids.

It was found necessary to vary the proportions of soap and tar-acids in dealing with the different fractions. The higher the boiling-point of the tar-acids the higher was the proportion of soap necessary to emulsify these.

Fractions No. 4 (205°—209° C.), 5 (209°—213° C.), 6 (213°—218·5° C.), 7 (218·5°—225·5° C.), and 8 (225·5°—240° C.) of the tar-acids were emulsified according to the following formula by the method described in Chapter V. :—

Tar-acid	.....	50
Castor oil	.....	20
Potash	.....	5
Water	.....	5; all by weight.

Fractions No. 9 (240°—274° C.) and 10 (274°—286° C.) were, however, emulsified by the following formula :—

Tar-acid	.....	50
Castor oil	.....	35
Potash	.....	8
Water	.....	7; all by weight.

The products were dark, translucent, homogeneous fluids, which were not very viscous and which were freely miscible with water. Those containing fractions 4, 5, 6 and 7 gave at first clear dilutions with distilled water, which very soon became milky. With tap-water the dilutions were immediately milky owing to precipitation of some of the castor-oil soap by the calcium and magnesium salts and consequent separation as a white emulsion of some of the tar-acids. The disinfectant-fluids containing Fractions No. VIII., IX. and

Potash ..... 8 Water ..... 7 (All by weight.)	11·0	2·3	15·3	3·0	·25%	·1%	1·5%	·75%	1%	·5%
V. Fraction X.— (274°-286° C.) Tar-acid..... 50 Castor-oil ..... 35 Potash ..... 8 Water..... 7 (All by weight.)	12·8	1·8	13·0	2·0	·25%	·1%	1·5%	·75%	3%	1·5%

TABLE XII.

Nature of Phenols Emulsified with Castor-oil Soap and Formula of Preparation.	Germicidal Power.				Concentrations recommended to be used in practice.					
	Staphylococcus pyogenes aur.		B. Typhosus.		For washing hands and sterilising instruments (absence of organic matter).		For disinfection of typhoid stools.		For disinfection of pus from empyemas.	
	Carbolic-acid co-efficient in the absence of feces.	Carbolic-acid co-efficient in the presence of 3 per cent. feces.	Carbolic-acid co-efficient in the absence of feces.	Carbolic-acid co-efficient in the presence of feces.	(Concentrations to be added to ½ their volume of stools or pus.)					
					At 20°C.	At 35°C.	At 20°C.	At 35°C.	At 20°C.	At 35°C.
T. Fraction IV.— (205°–209° C.) Tar-acid ..... 50 Castor-oil ..... 20 Potash ..... 5 Water ..... 5 (All by weight.)	3·7	2·2	6·8	2·4	1%	·5%	1·5%	·75%	—	—
U. Fraction V. and VI.— (mixed) (209°–218° C). Tar-acid..... 50 Castor-oil ..... 20 Potash ..... 5 Water..... 5 (All by weight)	5·5	2·8	7·2	3·1	·5%	·25%	1·5%	.75%	—	—
V. Fraction VII.— (218·5°–225·5° C.) Tar-acid..... 50 Castor-oil ..... 20 Potash ..... 5 Water ..... 5 (All by weight.)	5·7	3·6	9·5	3·0	·5%	·25%	1·5%	.75%	—	—
W. Fraction VIII.— (225·5°–240° C.) Tar-acid ..... 50 Castor-oil ..... 20 Potash..... 5 Water..... 5 (All by weight.)	8·6	2·4	14·1	3·2	·25%	·1%	1·5%	·75%	1%	·5%
X. Fraction IX.— (240°–274° C.) Tar-acid... ..... 50 Castor-oil ..... 35 Potash ..... 8 Water ..... 7 (All by weight.)	11·6	2·3	13·8	3·6	·25%	·1%	1·5%	·75%	1%	·5%
Y. Fraction X.— (274°–286° C.) Tar-acid..... 50 Castor-oil ..... 35 Potash ..... 8 Water..... 7 (All by weight.)	12·8	1·8	13·0	2·0	·25%	·1%	1·5%	·75%	3%	1·5%

X., however, immediately yielded milky dilutions with both distilled and tap-water owing to the greater insolubility of the constituent phenols, which on separation were emulsified by the soap.

These disinfectants prepared with castor-oil soap, unlike those containing soft soap, formed stable emulsions with distilled water, there being little deposition of tar-acid on standing. The emulsions with tap-water, however, were just a little less stable owing to the precipitation of some of the emulsificant (soap).

The effects of tap-water and of the age of the emulsions upon germicidal power are described later on in this chapter.

In Table XII. are given the results of the determinations of the germicidal powers of the preparations of the higher tar-acids emulsified by means of castor-oil soap.

From these results the following conclusions can be drawn :—

(i.) As was found in the case of preparations of the tar acids made with soft soap, the germicidal power of those made with castor oil soap increased with the boiling-points of the constituent tar acids.

(ii.) By comparing these results with those tabulated in table XI. it was found that the castor oil soap preparations of the higher tar acids containing a higher proportion of phenols exceeded the corresponding soft soap preparations in germicidal power. Further, the castor oil soap preparations gave much more stable emulsions with water.

(iii.) The depreciating effect of fæces upon germicidal power was much greater in the case of the preparations of tar acid fractions VIII., IX., and X., giving very milky emulsions with water, than in the case of the preparations of fractions IV. to VII., that gave milky emulsions less readily. The carbolic coefficients of the latter in presence of fæces were often equal to or even greater than those of the highest fractions. This shows that for disinfection in presence of fæces there is no advantage in employing preparations of the highest boiling-point tar acids.

(iv.) Although phenol exerted under the same conditions nearly equal germicidal powers upon B. typhosus and Staphylococcus, yet the carbolic acid co-efficients of these tar-acid preparations were greater in the case of B. typhosus than of Staphylococcus. This meant that the latter was more resistant to the germicidal action of these disinfectants than B. typhosus.

To this generalisation there were two exceptions. The carbolic acid coefficients of the preparations containing the two highest tar acid fractions, IX. and X., were the same, whether the organism was B. typhosus or Staphylococcus. This was an interesting case of selective disinfection and showed that Staphylococcus, compared with B. typhosus, was relatively more susceptible to the germicidal action of the highest tar acids than to that of the lower acids.

The Germicidal Power of Emulsified Crude Mixture of Tar Acids from Creosote-Oil.

The "crude tar acids" form the mixture of phenoloids obtained by extracting "creosote oil" with soda and decomposing the soda compounds of the tar acids with acids.

By the fractional distillation of this mixture of phenols the various fractions of tar acids are obtained, the bactericidal powers and methods of emulsification of which have been described above.

This mixture of tar acids was found to be best emulsified by means of castor oil soap. The method of emulsification is described in Chapter V. The proportions used were :—

Tar Acid	50
Castor Oil	20
Potash	5
Water	5

The product was a dark-coloured, homogeneous fluid, which gave a permanent pink emulsion with water. Its bactericidal powers under various conditions have been determined, and are set forth in Table XIII.

This preparation of the crude mixture of tar acids thus considerably exceeded phenol and the cresols in germicidal power. Its efficiency was greatly decreased by the presence of particulate organic matter.

B.—*The value of some of these disinfectants in the disinfection of pus.*

Since pus contains organic matter—in the particulate state as pus corpuscles, which frequently undergo fatty degeneration and are, therefore, rich in fat, and in the dissolved state as serum proteins—it was to be expected that its presence would greatly reduce the germicidal power of disinfectants. The pus employed in the experiments came from a case of empyæma. It contained no sporing organisms, but a large number of rod-shaped bacteria similar to B. coli and a few chains of cocci. The organisms grew rapidly on agar, forming a thick creamy growth. The total solids of the pus was first determined by drying a known weight of the well shaken pus over the wath-bath and, when most of the water was driven off, by adding a little absolute alcohol to facilitate the removal of the remaining water from the fatty mass. The pus was finally dried at 105° C.

The sample of pus contained 10 per cent. by weight of total solids. Its specific gravity was 1·016 at 15° C.

The method of determining germicidal power in the presence of the pus consisted in rapidly adding at one minute intervals known volumes of a concentration of a disinfectant to a series of 1·5 ccs. of pus to which varying volumes of water had been added. The volume of disinfectant added was such to make the total volume 5 ccs. The tubes were then subcultured in the way described in Chapter I.

As 1·5 ccs. of pus were always employed, the amount of solid matter present was always ·152 gms. in 5 ccs. of disinfectant, or 3 per cent. as in the case of the experiments with faeces. The results of the experiments are tabulated below (page 30).

From these results it is seen that the carbolic coefficients of the emulsified phenols in the presence of pus decrease as the series of higher tar acids is ascended, until the highest fraction (X.) is about as efficient as the cresols.

It, therefore, appears that the particulate organic matter in pus behaves as the particulate organic mater in fæces in reducing the germicidal value of emulsified disinfectants.

Table XIII.—Disinfectant Z.

Staphylococcus.		B. Typhosus.				Concentrations recommended for use in practice.					
						Disinfection in absence organic matter. Sterilisation of hands, instruments.		Disinfection in presence of fæces.		Disinfection of pus.	
In the absence of organic matter.		In the absence of organic matter.		In the presence of fæces.		2 volumes of concentration to be added to 1 of stool or pus.					
Carbolic co-efficient.	Concentration killing in 15 mins.	Carbolic co-efficient.	Concentration killing in 15 mins.	Carbolic co-efficient.	Concentration killing in 15 mins.	At 20° C.	At 35° C.	At 20° C.	At 35° C.	At 20° C.	At 35° C.
2·5	3·6 in 1000	6·8	1·1 in 1000	2·4	4 in 1000	1%	·5%	1·5%	·75%	1·5%	·75%

TABLE XIV.

DISINFECTANT.	Concentration of disinfectant required to disinfect the pus in 15 minutes.	Concentration of phenol required to disinfect the pus in 15 minutes	Carbolic Acid co-efficient.	Concentrations recommended for use in practice. One volume of the concentration of the disinfectant to be added to half its volume of pus.	
				At 20° C.	At 35° C.
E Crude Carbolic Acid (Cresols) emulsified with an equal weight of soft soap ...	8 in 1000	10 in 1000	1·2	3%	1½%
W Fraction VIII. Tar-acids (225·5°—240°C.) emulsified with castor oil	2 in 1000	12 in 1000	6·0	1%	½%
X Fraction IX. Tar-acids emulsified with castor oil	2·2 in 1000	11 in 1000	5·0	1%	½%
Y Fraction X. Tar-acids emulsified with castor oil	7·4 in 1000	11 in 1000	1·5	3%	1½%
Mixture of Crude Tar-acids emulsified with castor oil	4·0 in 1000	11 in 1000	2·7	1·5%	·75%

C.—*The influence of tap water upon the germicidal efficiency of disinfectants containing the higher tar acids.*

(*1*). *The effect of tap-water upon germicidal power in absence of organic matter.*

The determination of germicidal power was carried out by the method already described, except that tap-water was employed in making up the dilutions. Control experiments with distilled water were carried out at the same time.

The results are tabulated below :—

TABLE XV.

Disinfectant.	Organism.	Dilutions made with distilled water.		Dilutions made with tap-water.	
		Carbolic-acid co-efficient.	Concentration killing in 15 mins.	Carbolic-acid co-efficient.	Concentration killing in 15 mins.
Phenol	Staphylococcus	—	9·5 in 1000	—	12·0 in 1000
W. Fraction VIII.— Emulsified with Castor-oil Soap	,,	8·6	1·1 in 1000	9·2	1·3 in 1000
Y. Fraction X.— Emulsified with Castor-oil Soap	B. Typhosus ..	13·0	·58 in 1000	14·6	·65 in 1000
Phenol	,,	—	7·5 in 1000	—	9·5 in 1000
Y. Fraction XI.— Emulsified with Castor-oil Soap	Staphylococcus	12·8	·78 in 1000	12	1 0 in 1000
Phenol	,,	—	10 in 1000	—	12 in 1000
V. Fraction VII.— Emulsified with Castor-oil Soap	Staphylococcus	5·7	1·4 in 1000	6·1	1·8 in 1000
Phenol	,,	—	8 in 1000	—	11 in 1000
X. Fraction IX.— Emulsified with Castor-oil Soap	Staphylococcus	1·6	·82 in 1000	9·6	1·1 in 1000
Phenol	,,	—	9·5 in 1000	—	11·4 in 1000

The presence of tap-water, therefore, appears to decrease, by 15 to 30% the germicidal power of the higher tar acids. Depreciation to nearly the same extent was observed with phenol. This does not seem to be experimental error, as the results are consistent (see Table XV.) The explanation is not obvious.

(*ii.*) *The effect of tap-water upon the stability of the emulsions.*

As disinfectants will in practice be diluted with tap-water, it is important that the emulsion shall be stable when

so diluted. Many emulsified disinfectants on the market show considerable deposition when diluted with tap-water, the deposition varying in extent according to the hardness of the water.

The sample of tap-water used in the experiments came from the Thames Valley.

In the experiments carried out to determine the influence upon stability of the emulsions dilutions of various disinfectants were made with distilled and tap-water and their

TABLE XVI.

Disinfectant	W. Fraction VIII. emulsified with castor-oil soap.	Y. Fraction X emulsified with castor-oil soap.	V. Fraction VII. emulsified with castor-oil soap.
Organism ..	Staphylococcus.	B. Typhosus.	Staphylococcus.
Carbolic Acid co-efficient determined with *distilled* water and *before* standing	8·6	12·6	5·7
Carbolic Acid co-efficient determined with *distilled* water and *after* standing	9·2	10·0	6
Time of standing ..	48 hrs.	96 hrs.	48 hrs.
Carbolic Acid co-efficient determined with *tap-water* and *before* standing	9·2	14·6	5.64
Carbolic Acid co-efficient determined with *tap-water* and *after* standing	6·5	12·0	5·4
Time of standing ..	48 hrs.	96 hrs.	48 hrs.

germicidal power was immediately determined. The dilutions were then allowed to stand several days. After that time there was usually a little separation of tar acid from emulsions made with both distilled and tap-water and the germicidal powers of the supernatant liquids were determined, shaking up being carefully avoided.

The results of these experiments are set forth in Table XVI.

The emulsions of the higher phenols in some cases, therefore, tended to fall in germicidal power on standing a few days and the depreciation was generally greater in the case of the emulsions made with tap water than of those made with distilled-water.

These facts point to the advisability of either employing in disinfection freshly made emulsions of the higher phenols or shaking up the emulsions before use when they have been made for some time.

V.—On the Choice of Emulsificants for the Higher Tar Acids.

Soft soap was found to be an inefficient emulsificant for the higher tar acids. A search was therefore made for substances superior to soft soap in emulsifying power. They were at first looked for amongst proteins and carbohydrates.

Aqueous solutions of egg-albumen were useless for the emulsification of the phenols, because the protein was immediately coagulated.

Casein, on the other hand, was not precipitated by the phenols and possessed considerable emulsifying power. A 6 per cent. solution of casein was used for emulsification and was prepared by dissolving precipitated casein in a 1·5 per cent. aqueous solution of sodium carbonate with the aid of heat.

Equal volumes of the casein solutions and of the tar acids were triturated together and the tar acids were thereby emulsified, forming a white emulsion. These emulsions, however, were not readily miscible with water and they were also less permanent than emulsions of the same tar acids with soft soap.

Saturated aqueous solutions of glucose, solutions of agar-agar, and 1 per cent. solutions of starch possessed no emulsifying properties towards the higher tar-acids.

Mucilages of gum acacia and gum ghatti, however, were able to emulsify the higher phenols, but the resulting emulsions, although readily miscible with water, were not so permanent as those made with soft soap.

The emulsifying agents of protein and carbohydrate nature employed were thus not as efficient as soft soap, and a further examination of the soap class of emulsificant was made. Resin soap, although it was used as an emulsificant of the cresols, could not permanently emulsify the higher phenols. It had a further practical disadvantage, insomuch as admixtures of resin soap and the high boiling point phenols were of an excessively viscous nature.

Castor-oil soap, however, was found to be an efficient emulsificant for the higher tar acids. Castor oil and tar acid were mixed and heated in the water-bath; the potash dissolved in water was added to the hot mixture and heating was continued until saponification was complete, this stage being reached when there was no longer a fatty scum produced when a drop of the mixture was diluted with water. The products were dark-coloured fluids, which were not very viscous and which gave stable emulsions with water.

VI.—Germicidal Power and Cost in the Case of the Coal-Tar Disinfectants.

The costs of the various preparations are best compared in terms of their carbolic coefficients. The costs of the amounts of the disinfectants equivalent in germicidal power to 10 lbs. of carbolic acid have been determined. These are obtained by dividing the price per 10 lbs. by the carbolic coefficient.

Soft soap quality P.C., which is 17s. per cwt. or about 2d. per lb., is much cheaper than the B.P. quality. As will be shown in Chapter VII., there is no advantage in using the more expensive quality and it has therefore been assumed in calculating the costs that the former would be employed.

For the purpose of calculation the following costs of other ingredients used in the manufacture of the disinfectants have been used :—

Resin	2d. per lb.
Potash (purified by 87 per cent. alcohol)	1s.9d. per lb.
Castor oil (bleached and filtered)	9d. per lb.
Phenol (pure crystals)	£2 per 10 lbs.
B.P. liquefied phenol (containing 9-10% water)	10s. per 10 lbs.
Crude carbolic acid	1s. to 1s. 7½d. per gal. [1½d. per lb.]
Thymol	7s. per lb.
Crude mixture of tar acids	3s. per gallon

The separated fractions of the higher tar acids (Fractions I. to X.) are sold at 2s. 6d. per gallon (about 3d. per lb. on the condition that all the fractions are simultaneously bought. If, however, a selection of one or more of the fractions were made the price would be much higher. The costs are worked out below on the assumption that no selection would be made.

In the following tables are given the cost of making the various preparations and also the costs of the amounts equivalent in germicidal power to 10 lbs. of phenol. Since germicidal power depends so much upon the conditions of disinfection, the costs of employing the various disinfectants for different purposes have been made out.

The following conclusions are drawn from these results :—

(i.) It is more economical to use the resin-soap preparations* of crude carbolic acid than those containing soft-soap and the latter rather than the castor-oil soap preparations. The disinfectants containing resin-soap have the disadvantage, however, of producing milky dilutions with water while the dilutions of the soft-soap preparations are nearly clear.

(ii.) For the disinfection of Staphylococcus* in absence of organic matter, of typhoid stools and pus it is more economical to employ crude carbolic acid emulsified with soft-soap than the crude mixture of tar-acids preparation.

(iii) It is more economical to employ the higher boiling-point phenols (Fractions IV. to X.) for disinfection in the absence of organic matter than the cresols. This advantage increases with the boiling points of the tar acids, but its practical value is decreased by the great expense incurred in obtaining a large amount of any *separate* fraction. There is no such advantage in the presence of fæces ; in fact, under these conditions it is usually more economical to use the cresols.

(iv) The use of the thymol preparation is more costly than that of crude carbolic acid emulsified with soft soap, especially in the presence of organic matter.

---

*For practical purposes the cost of disinfecting Staphylococcus rather than B. typhosus in absence of organic matter have been compared on account of the higher resistance of the former organism to the action of disinfectants.

TABLE XVII.

	Conditions	Carbolic-	Price per	Cost of an amount of disinfectant equiva-
	Disinfection of B. Typhosus in presence of fæces	1·8	—	1·39
K Tar-acid Fractions I., II., and III. - 50 Resin - 25 Potash - 6 Water - 5 all by weight	Disinfection of Staphylococcus in absence of organic matter	3·0	3/2	1·05
	Disinfection of B. Typhosus in absence of organic matter	3·6	—	0·88
	Disinfection of B. Typhosus in presence of fæces	2·6	—	1·22
T Fraction IV. (208-209°c) 50 Castor oil 20 Potash - 5 Water - 5 all by weight	Disinfection of Staphylococcus in absence of organic matter	3·7	—	1·23
	Disinfection of B. Typhosus in absence of fæces	6·8	—	0·67
	Disinfection of B. Typhosus in presence of fæces (10% solids)	2·4	—	1·91

TABLE XVII.—*Continued.*

	Conditions	Carbolic-	Price per	Cost of an amount of disinfectant equiva-
	sence of fæces			
	Disinfection of pus	1·5	—	3·60
Thymol - 50 Castor-oil 20 Potash - 5 Water - 5 by weight.	Disinfection of Staphylococcus in absence of organic matter.	23	£2 7 11	2·08
	Disinfection of B. Typhosus in absence of organic matter.	30		1·6
	Disinfection of B. Typhosus in presence of fæces.	8·8		5·44
Mixture of crude tar-acids - 50 Castor-oil 20 Potash - 5 Water - 5 by weight	Disinfection of Staphylococcus in absence of organic matter.	2·5	0 5 0	2·00
	Disinfection of B. Typhosus in absence of same.	6·8		0·73
	Disinfection of B. Typhosus in presence of fæces (3 %)	2·4		2·08
	Disinfection of pus	2·7		1·82

TABLE XVII.

Disinfectant.	Conditions of Disinfection.	Carbolic-acid co-efficient.	Price per 10 lbs. of disinfectant.	Cost of an amount of disinfectant equivalent in germicidal efficiency to 10 lbs. of pure phenol at £2.
				s.
B C D Tar-acid Fractions, I. II. III. emulsified with an equal weight of soft-soap.	Disinfection of Staphylococcus in absence of organic matter	1·6	2/1	1·30
	Disinfection of B. Typhosus in absence of organic matter	3·0	—	0·70
	Disinfection of B. Typhosus in presence of fæces (10% solid matter)	1·8	—	1·15
E Crude carbolic-acid, emulsified with an equal amount of soft-soap.	Disinfection of Staphylococcus in absence of organic matter	·9	1/8	1·75
	Disinfection of B Typhosus in absence of organic matter	1·5	—	1·10
	Disinfection of B. Typhosus in presence of fæces	1·1	—	1·50
	Disinfection of pus	1·2	—	1·66
H Crude carbolic-acid 50 Castor oil 20 Potash - 5 Water - 5 all by weight	Disinfection of Staphylococcus in absence of organic matter	1·6	3/9	2·34
	Disinfection of B. Typhosus in absence of organic matter	3·6	—	1·04
	Disinfection of B. Typhosus in presence of fæces	2·2	—	1·70
I Tar-acids, Fractions I., II. or III. - 50 Castor oil 20 Potash - 5 Water - 5 all by weight	Disinfection of Staphylococcus in absence of organic matter	2·9	4/6	s. 1·55
	Disinfection of B. Typhosus in absence of organic matter	5·2	—	0·86
	Disinfection of B. Typhosus in presence of fæces	3·4	—	1·32
J Crude carbolic-acid 50 Resin - 25 Potash - 6 Water - 5 all by weight	Disinfection of Staphylococcus in absence of organic matter	1·9	2/6	1·31
	Disinfection of B. Typhosus in absence of organic matter	2·5	—	1·00
	Disinfection of B. Typhosus in presence of fæces	1·8	—	1·39
K Tar-acid Fractions I., II., and III. - 50 Resin - 25 Potash - 6 Water - 5 all by weight	Disinfection of Staphylococcus in absence of organic matter	3·0	3/2	1·05
	Disinfection of B. Typhosus in absence of organic matter	3·6	—	0·88
	Disinfection of B. Typhosus in presence of fæces	2·6	—	1·22
T Fraction IV. (208-209°c) 50 Castor oil 20 Potash - 5 Water - 5 all by weight	Disinfection of Staphylococcus in absence of organic matter	3·7	—	1·23
	Disinfection of B. Typhosus in absence of fæces	6·8	—	0·07
	Disinfection of B. Typhosus in presence of fæces (10% solids)	2·1	—	1·91

TABLE XVII.—*Continued.*

Disinfectant.	Conditions of Disinfection.	Carbolic-acid co-efficients.	Price per 10 lbs.	Cost of an amount of disinfectant equivalent in germicidal efficiency to 10 lbs. of pure phenol.
U Fractions V and VI (209°-218·5°c) 50 Castor oil 20 Potash - 5 Water - 5 all by weight	Disinfection of Staphylococcus in absence of organic matter	5·5	4/7	0·83
	Disinfection of B. Typhosus in absence of fæces	7·2	—	0·64
	Disinfection of B. Typhosus in presence of fæces	3·1	—	1·48
V Fraction VII. (228.5°—225.5°) 50 Castor-oil 20 Potash - 5 Water - 5	Disinfection of Staphylococcus in absence of organic matter	5.7	4/7	0·80
	Disinfection of B. Typhosus in absence of organic matter	9·5	—	0·48
	Disinfection of B. Typhosus in presence of fæces	3·0	—	1·53
W Fraction VIII. (225·5°—240°C) 50 Castor-oil 20 Potash - 5 Water - 5	Disinfection of Staphylococcus in absence of organic matter	8·6	4/7	0·53
	Disinfection of B. Typhosus in absence of organic matter	14·1	—	0·32
	Disinfection of B. Typhosus in presence of fæces	3·2	—	1·43
	Disinfection of pus	6·0	—	0·76
X Fraction IX 50 Castor-oil 35 Potash - 8 Water - 7 All by weight	Disinfection of staphylococcus in absence of organic matter	11·6	5/5	0·47
	Disinfection of B. Typhosus in absence of fæces	13·8	—	0·33
	Disinfection of B. Typhosus in presence of fæces	3·0	—	1·48
	Disinfection of pus	5·0	—	1·08
Y. Fraction X. 50 Castor-oil 35 Potash - 8 Water - 7	Disinfection of staphylococcus in absence of organic matter	12·8	5/5	s. 0·42
	Disinfection of B. Typhosus in absence of fæces	13·0	—	0·42
	Disinfection of B. Typhosus in presence of fæces	2·0	—	2·60
	Disinfection of pus	1·5	—	3·60
Thymol - 50 Castor-oil 20 Potash - 5 Water - 5 by weight.	Disinfection of Staphylococcus in absence of organic matter.	23	£2 7 11	2·08
	Disinfection of B. Typhosus in absence of organic matter.	30		1·6
	Disinfection of B. Typhosus in presence of faces.	8·8		5·44
Mixture of crude tar-acids - 50 Castor-oil 20 Potash - 5 Water - 5 by weight	Disinfection of Staphylococcus in absence of organic matter.	2·5	0 5 0	2·00
	Disinfection of B. Typhosus in absence of same.	6·8		0·73
	Disinfection of B. Typhosus in presence of fæces (3 %)	2·4		2·08
	Disinfection of pus	2·7		1·82

VII.—Observations Bearing on the Alkalinity, Toxicity, Irritating Properties and Action on Rubber of the Preparation of Cresols and Higher Phenols.

1. *The alkalinity of the preparations.*

When the soap preparations are diluted with water, some of the soap is hydrolysed with production of fatty acid and potash :—

$$H_2O + K\overline{X} \rightleftarrows H\overline{X} + KOH$$

Soap — Fatty acid

The free alkali so formed will for the most part combine with the phenols, forming phenolates. The phenolates are also hydrolysed in aqueous solution :—

$$H_2O + C_6H_5OK \rightleftarrows C_6H_5OH + KOH$$

but as there is a large excess of phenol or cresol present this will be negligible.

(*i.*) *The alkalinity of the soft soap preparations.*

B.P. soft soap should not give a reaction for free caustic alkali when an alcoholic solution of phenol-phthalein is dropped upon a fresh surface of the soap. The soap being moist, the absence of coloration indicates that their is no free alkali hydroxide, and also no free carbonate, borate, and silicate. [If the soap be somewhat dry, no coloration proves the absence of free hydroxide, but not necessarily of free carbonate, borate, and silicate.] A sample of Price's B.P. soap undried gave no evidence of the presence of free alkali.

Price's P.C. soft soap has been used in the above experiments for emulsifying the cresols instead of the B.P. soap on account of its cheapness. It gave, however, a pink coloration with phenol-phthalein, indicating the presence of free alkali.

The total alkali of these two samples was determined in the following way :—10 gms. of the soap were dissolved in hot water and excess of standard acid was added in known amount. The mixture was heated, stirred and then cooled in an ice bath, so that the fatty acids formed by the decomposition of the soap were solidified. They were filtered off and well washed with warm water, and the washings added to the filtrate. The combined filtrate was then titrated with standard alkali in order to determine the amount of acid unused in precipitating the acids and in combining with all the alkali present. This amount subtracted from the original amount of acid taken gave the quantity of acid required to unite with all the alkali—free and combined—present in the soap, and the alkali equivalent of this amount of acid was expressed in terms of potassium oxide, K_2O.

The total alkali content of B.P. soft soap was 8·23 per cent., this being the mean of three determinations.

The mean of three estimations of the total alkali content of P.C. soap was 9·47 per cent. The small difference in the alkali contents of the two soaps suggested that there was no advantage in employing the B.P. soap instead of the cheaper quality in the preparation of disinfectants.

The following calculation shows that this is so. Since the cresol suspensions prepared with P.C. soft soap contained 50 per cent. of the latter, their alkali content would be 4·73 per cent. Two per cent. dilutions with water of these cresols—

i.e., the strength commonly employed in the case of these disinfectants—would, therefore, contain ·09 per cent. total alkali.

If the entire alkali in the 2 per cent. dilutions were in the free state it would amount to about 1·50th normal, but only a fraction of the total alkali in a soap dilution is free, and in the presence of the cresols free alkali could not be detected with phenol-phthalien—*i.e.*, its concentration was less than $10^{-5\cdot5}$ normal.

(*ii.*) *The alkalinity of the resin soap and castor oil soap preparation.*

In these preparations a known amount of alkali was introduced since the soap was synthesised from fat in presence of tar acid.

In the following table are given the alkali contents of the various preparations, which are classified below according to their formulæ :—

TABLE XVIII.

Formula.	Alkali-content % of preparation (KOH)	Alkali-content (KOH) of 2 % dilution.
I. Tar-acid 50, Castor-oil 20, Potash 5, Water 5 ..	6·25 %	·125 %
II. Tar-acid 50, Castor-oil 35, Potash 8, Water 7 ..	8 %	·160 %
III Tar-acid 50, Resin 25, Potash 6, Water 5 ..	7 %	·140 %

If the entire alkali in the 2 per cent. dilutions were in the free state it would amount to a concentration of from 1-33rd to 1-42nd normal, according to the formula of the preparation.

It was found, however, that the concentration of free alkali in the 2 per cent. dilutions of the castor oil soap and resin soap preparations only amounted to 1-170th normal. This strength of alkali can just be tasted and causes slight smarting when applied to the conjunctiva.

The dilutions with water of the various soap emulsions of the phenols, therefore, contained very little free alkali.

(*ii.*) *Toxicity.*

The tar acids exert a poisonous action when injected or taken orally. The minimum lethal dose of carbolic acid for man appears to be 15 gms., but fatal poisoning by phenol has been known to occur as a result of its application to the unbroken skin and also of its injection into the cavities of abscesses.

Tollens (Archiv f. Experiment. Path. u. Pharm., Vol. 52, p. 220) showed that fatal doses of carbolic acid and of the isomeric cresols were nearly the same. His experiments were carried out upon cats, mice, and frogs.

He also showed that there was no difference between the toxicities of aqueous solutions and soap emulsions of phenol

of the same strength. This was also true of the cresols. A cresol soap emulsion was, therefore, equal in toxicity to a phenol soap emulsion. For example, Liquor cresoli saponatus was just as toxic as the corresponding phenol preparation. Although the toxicities to animals of phenol and the cresols are equal, the latter are considerably superior to phenol in germicidal power. This means that the use of cresol preparations is less dangerous than that of phenol preparations, because more dilute solutions of the cresols can be used for disinfecting purposes.

The fact that the soap emulsions are no more toxic than aqueous solutions is also of practical importance, because of the advantages in employing the cresols in the emulsified state as disinfectants.

The poisonous properties of the cresols are also indicated by the fact that many cases of poisoning with lysol have recently occurred in Germany. Lysol has, however, been employed in the treatment of cerebro-spinal meningitis by the injection of its 1 per cent. solution into the spinal cord without any poisonous effects.

There are cases, too, that show that the cresols have not always so great a toxicity as carbolic acid. Thus, 4 ozs. of Jeyes' fluid and 9 ozs of creolin have been taken by the mouth and there has been complete recovery from their toxic effects after a period of unconsciousness. Such results are probably due to the de-emulsification of the higher tar acids in the stomach and their very slow absorption, owing to their slight solubility. Survival would have been unlikely in the case of carbolic acid.

The following information concerning the toxicity of the higher tar-acids is taken from Lewin's "Lehrbuch der Toxikologie, 2*te* Auft. 1897," pp. 215 and 361.

A dose of 2·5 gms. of creosote can kill cats and rabbits, the corresponding minimum lethal dose per kilogram body-weight being about 0·7 gm. The minimum lethal dose per kilo. of phenol and cresol is ·09 gm. for cats (Tollen's l.c.), so that creosote is much less poisonous than these substances. One fifth of a gram of creosote can kill pigeons, and dogs are killed by 6 to 7 gms. Cases are recorded in which a dose of 7 gms. of creosote has fatally poisoned a man, and doses beginning at 20 drops and progressively increasing to 1·8 gm. have killed a child.

Thymol is also poisonous, as 3 to 4 gms. of it can kill rabbits by injection and 5 to 6 gms. can kill the same when given orally. Since only a dose of 2½ gms. of creosote is necessary to kill rabbits, it is seen that thymol is less poisonous than creosote, and, therefore, much less poisonous than phenol and cresols.

Cases are recorded in which a dose of from 6 to 10 gms. of thymol has caused toxic symptoms, such as vomiting, collapse, etc., in man.

There is, therefore, some evidence showing that the higher tar-acids are less poisonous than phenol and the cresols. The dangers attending the use of the higher tar-acids are further decreased by their high germicidal power, which permits of their employment as disinfectants in small concentrations.

(iii.)—*The irritating properties of the tar acids.*

The concentrated preparations when spilt upon the skin are very caustic and produce bad wounds. Two per cent.

dilutions of them in water however, have no immediate irritant effect upon the normal skin, and at this dilution the preparations exert a reliable germicidal action.

Two per cent. dilutions when placed upon the tongue produce a stinging sensation.

0·25 per cent. dilutions of all the fractions of the tar acids produce slight stinging when placed upon the conjunctiva.

No striking differences could be made out between phenol, cresol, and a higher fraction of tar acid (No. ix.) boiling at 270°—286° C. in regard to their stimulating properties on the conjunctiva.

0·25 per cent. of phenol or cresol would not effect a reasonable germicidal effect, but 0·1 per cent. of the preparations of the higher fractions, with their much higher germicidal power, could be usefully employed. More concentrated emulsions are not suitable for sensitive mucous membranes.

(iv.)—*The action of the coal-tar disinfectants upon rubber.*

Pieces of rubber tubing in good condition were immersed for twenty days in 2 per cent. solutions of crude carbolic acid emulsified with resin and soft soap and of a higher tar-acid fraction, No. ix., emulsified with castor-oil soap.

The immersion in these dilutions had no effect upon the rubber other than that of softening, which was no greater than the softening effect produced by immersion in water for the same length of time.

It was noticed that the emulsions of the higher tar-acid in which the rubber had been immersed had considerably increased in opacity while control emulsions with no rubber had only decreased slightly owing to a small deposition of tar acid. This showed that some of the latter had been absorbed by the rubber, which would therefore behave as particulate organic matter in decreasin germicidal power.

The concentrations of the various preparations recommended for disinfecting stools can be recommended for disinfecting rubber instruments.

Preparations for Disinfection of Hands, Instruments, &c.

The following preparations are recommended for disinfection of hands, instruments, pus, etc. They possess a reliable germicidal power, are readily manufactured, are considerably cheaper than phenol, and for the same germicidal power are less poisonous.

The fact that rise in temperature greatly increases germicidal power is of importance in practice. The concentrations recommended below for disinfection at ordinary temperatures may be halved if the disinfection is conducted at 35° C. or above.

(I). *Crude Carbolic Acid (Cresols), emulsified with Soft Soap.*

(Disinfectant E. Table III., Chapter II.

Crude carbolic acid, which consists chiefly of cresols, can be obtained from gas companies or disinfectant manufacturers.

Take equal weights of crude carbolic acid and soft soap (Price's P.C. quality); heat them together at 80° C. until all the soap is dissolved and filter the product. The preparation is a dark colored homogeneous fluid readily miscible with tap-water, forming a slightly turbid liquid.

Dilution recommended for hands and instruments	2%
Dilution recommended for disinfection of stools, pus, and other particulate organic matter, on the assumption that for one volume of stools, etc., at least two volumes of the diluted disinfectant be added...	3%

The costs of materials to make one gallon of this preparation is 1s. 8d., and that of an equal amount of B.P. carbolic acid is 10s. The disinfectant is therefore much cheaper than phenol, and at the same is equal to it in germicidal power and is less poisonous.

This preparation is identical with the Liquor creosoli saponatus, official in the German, Japanese, and Swedish Pharmacopœias, and with the Cresolum saponatum of the Belgic and Swiss Pharmacopœias.

(II.) *Crude Mixture of Tar Acids from Creosote Oil, emulsified with Castor-oil Soap.*

(Disinfectant Z. Table XIV., Chapter IV.)

The "crude mixture of tar acids" contains cresols and their higher homologues, together with some carbolic acid, and is obtained by the extraction of creosote oil with soda and acidification of the extract.

Creosote oil is the fraction collected between 230° C. and 270° C. in the distillation of coal tar.

The "crude mixture of tar acids" can be obtained from gas companies and disinfectant manufacturers.

Take 50 parts by weight of this mixture of tar acids and 20 parts of castor oil (bleached and filtered oil of commerce), mix well and heat to 90° C., add a solution of 5 parts of potash in 5 parts of water, and heat and stir until a drop of the mixture gives a pink emulsion with tap water with no fatty scum.

The product is a dark-coloured fluid, miscible with water. giving a stable pink emulsion.

Dilution recommended for hands and instruments	1%
Dilution recommended for disinfection of stools, pus, and other particulate organic matter, on the assumption that for one volume of stools, etc., at least two volumes of the diluted disinfectant be added	1·5%

The cost of the materials to make one gallon of the disinfectant is 5s. and is therefore much greater than that of one gallon of the crude carbolic acid preparation (1s. 8d.). The crude tar-acids disinfectant is both in the absence and presence of organic matter, however, superior to the crude carbolic-acid preparation in germicidal power, so that the cost per germicidal unit of the two preparations is approximately the same, Furthermore, the disinfectant made with the crude mixture of tar acids is considerably less poisonous than the crude carbolic-acid preparation, and this advantage is increased by the fact that, owing to its higher germicidal power, the concentrations of the former preparation recommended to be used in practice are much smaller than those of the latter.

The emulsions of the crude tar-acids disinfectant with tap water deposit a little tar acid on standing, so that it is important to employ its freshly-made dilutions or to shake thoroughly long-standing ones.

I desire to express my best thanks to Dr. C. J. Martin, F.R.S., for giving me the opportunity of conducting this investigation and also for help and advice in the course of the work, and to Messrs. Burt, Boulton, and Heywood for supplying samples of various tar acids.

[Reprinted from THE LANCET, Nov. 4, 1911, Vol. II., p. 1266.]

EXPERIMENTS ON THE CAUSATION OF BERI-BERI.

(Preliminary Communication.)

By E. A. COOPER, B.Sc.,

and

CASIMIR FUNK, Ph.D.

(From the Lister Institute of Preventive Medicine.)

The conclusion that beri-beri was due to a diet in which rice bulked too largely was arrived at by Wernich,[1] Van Leent,[2] and Takaki.[3] Acting upon this conclusion the latter, by modifying the diet of the sailors, has succeeded in practically eradicating the disease from the Japanese Navy. The vast amount of epidemiological evidence collected by Braddon[4] showed conclusively that beri-beri was associated with the continuous consumption of a diet composed too exclusively of decorticated (polished) rice. Braddon's observations brought out the further important point that natives, whatever their race, consuming rice which had been parboiled previous to removal of the pericarp (such as that used by the Indian Tamils) were not in danger of contracting the disease. One difference between these rices consists in the amount of subpericarpal layer remaining on the grains after the various manipulations to which they have been subjected prior to consumption.

An important fact for the investigation of beri-beri was the discovery of Eykman[5] that a polyneuritis in birds (fowls and pigeons) analogous to the human beri-beri is induced by a diet of polished rice, whereas fowls fed on undecorticated rice remained healthy. Eykman also showed that the latter variety of rice could cure fowls suffering from polyneuritis induced by a diet of the former. The next advance was due to Grijns,[6] who showed that the addition of pericarp and

subjacent layers removed during decortication (polishings) to the diet of decorticated rice prevented the occurrence of polyneuritis. Neither Eykman nor Grijns adopted the simpler interpretation of their facts that beri-beri was a disease due to the deficiency of some essential dietetic constituent in the decorticated rice, but suggested that the endosperm contained some poison, the antidote to which occurred in the cortical layers removed by polishing.

The solution of the problem has been materially advanced by the work of Fraser and Stanton.[7] These authors having confirmed the preventive and curative results obtained with polishings sought to separate from them the active substance. They extracted the polishings with 0·3 per cent. HCl and precipitated the extract with alcohol. By this means the greater portion of the phosphorus was removed in the form of phytin. By the suitable addition of this phytin to a diet of polished rice they failed to prevent in birds the occurrence of polyneuritis. The alcohol extract, however, possessed protective properties. The alcoholic extract contained proteins, but these were found to be devoid of action. The inactivity of the alcohol-soluble proteins has since been confirmed by Chamberlain and Vedder,[8] who found that the dialysate of the alcohol-soluble fraction contained the active substance.

Schaumann[9] has recently published a comprehensive study of the subject containing the results of many interesting and important experiments undertaken during the past three years. Schaumann's results may be briefly summarised as follows: 1. Inorganic phosphates had no curative properties. 2. Phytin, which had been credited by Aron[10] with some preventive properties, gave entirely negative results. 3. Complex mixtures, such as yeast, rice polishings, Katjang-idjoe beans, if administered in sufficient quantity to pigeons far advanced in the disease, produced a cure. 4. Although an impure commercial sample of yeast-nucleic acid cured the disease in 3 out of 14 cases (pigeons), it was not possible to obtain conclusive results with all kinds of simple phosphorus-containing substances, such as lecithin, glycerophosphates, and phospho-proteins. For these reasons Schaumann suggests (*l. c.* p. 347) that phosphorus compounds can only exercise curative effects when acting in conjunction with certain other substances.

With this we conclude the *résumé* of the more important work

on the problem of beri-beri and pass to the consideration of the results of our own experiments.

We have repeated feeding experiments of former workers upon birds (fowls and pigeons) with the various kinds of rice and have confirmed them, and have also confirmed the preventive action of rice bran (polishings). Experiments with an exclusive diet of various pure carbohydrates—starch, inulin, cane sugar, and dextrin—have been carried out, and it was found that such diets induced polyneuritis with the usual symptoms. These results disprove any intoxication hypothesis concerning beri-beri in birds. A daily ration of 0·5 gramme phytin administered to pigeons fed on white rice did not prevent polyneuritis, and attempts to cure polyneuritis by the addition of phytin, edestin, casein, and egg-yolk were unsuccessful. As the latter two substances are relatively rich in organically combined phosphorus the negative results are important. One gramme of dried pressed yeast per day prevented the development of polyneuritis in fowls, and we were also able to confirm the curative properties of pressed yeast. Yeast-juice obtained by squeezing the ground cells with kieselguhr in a press was found to exert a curative effect like the original yeast. *We have also found that pressed yeast hydrolysed with 20 per cent. sulphuric acid for 24 hours still retained its curative properties.* As all organic phosphorus compounds are completely hydrolised under such conditions, and phosphoric acid has been shown to be without influence, it is very improbable that the polyneuritis is the result of a deficiency in phosphorus compounds.

After confirming the results of Fraser and Stanton concerning the beneficial effects of the alcoholic extract of rice polishings, we precipitated a water solution of the extract by means of phospho-tungstic acid, and found that the active substance is precipitated entirely by this reagent and that the filtrate is completely inactive.

The curative effect of the precipitate decomposed by baryta in the usual way, which is wholly devoid of phosphorus and also free from carbohydrates or protein groups, was tried upon ten pigeons; it was found to be extraordinarily active and cured the birds even when they appeared moribund, striking improvement occurring in from three to ten hours. In several instances birds which were completely paralysed and supposed to be dying in the evening were found next morning with but some trace of lameness.

Polishings appear to contain only a very small amount of the active substance, and for further progress a very large quantity of polishings must be dealt with.

In conclusion, it appears that the polyneuritis induced in birds by a diet of decorticated and polished rice is not due to the deficiency of phosphorus compounds, but to the absence of some substance not of protein nature, devoid of phosphorus, soluble in water and acidulated alcohol, dialysable, and precipitated by phospho-tungstic acid, and the precise nature of which is being at present investigated by one of us (C. F.).

We are glad to have this opportunity of expressing our best thanks to Dr. C. J. Martin, F.R.S., Director of the Lister Institute, for drawing our attention to this subject, and for help and advice in the course of the investigation; and to Dr. Leonard Braddon of Seremban, Malay States, to whom we are indebted for kindly furnishing an adequate supply of different kinds of rice and rice polishings.

Bibliography.—1. Wernick: Geographisch-Medicinische Studien, Berlin, 1878. 2. Van Leent: Geneeskundig Tijdschrift voor Nederlandsch-Indie, 1880. 3. Takaki: Prevention of Kakke in Japanese Navy, Sei-i-Kwai, 1885, 1886, 1887. 4. Braddon: The Cause and Prevention of Beri-Beri, London, 1907. 5. Eykman: Virchow's Archiv, 148 (1897); Geneeskundig Tijdschrift voor Nederlandsch-Indie, 36 (1896). 6. Grijns: Ibid., 41 (1901) and 49 (1909). 7. Fraser and Stanton: The Lancet, March 12th, 1910, p. 733; Studies from the Institute for Medical Research, Federated Malay States, No. 12. The Etiology of Beri-Beri, 1911. 8. Chamberlain and Vedder: The Philippine Journal of Science, June, 1911, vol. vi. 9. Schaumann: Die Etiologie der Beri-Beri unter Berücksichtigung des Gesamten Phosphorstoffwechsels, Archiv für Schiffs- und Tropen-hygiene (1910), 14, Beiheft 8. 10. Aron and Hoeson; The Philippine Journal of Science, vol. v., February, 1910.

On Some Stages in the Life-History of Leptomonas muscæ domesticæ, with some remarks on the Relationships of the Flagellate Parasites of Insects.

By

J. S. Dunkerly.

With Plate 31.

In an endeavour to examine the biflagellate character of Herpetomonas as described by Prowazek (**20**), I have dissected and examined a number of house-flies. At first Musca domestica was investigated, as I had supposed that this was the animal indicated by the word "Stubenfliege." In this country, however, M. domestica does not seem to be commonly infected, as I was unable to find the parasite in it, and Hewitt (**6**), who examined a good number of these flies, was similarly unsuccessful. In the smaller house-fly, Homalomyia canicularis, flagellate parasites were found to be present, but Hewitt had confined himself to M. domestica. Still, the infections were very rare. I examined these flies taken in three distinct localities: Chelsea and Wandsworth in London, and Benfleet in Essex. Parasites were found in flies from each place, but always in a low percentage, about 4 per cent., of the flies examined. Other species of flies[1] have been examined, but not in large numbers, so that it is not surprising that no Herpetomonads have been met with in them as yet.

[1] I take this opportunity of expressing my thanks to Mr. Austen, who kindly assisted me in the identification of these flies.

I will first describe the forms met with by me in Homalomyia canicularis, and afterwards discuss their significance. (1) In the midgut (ventriculus) of two flies were seen some large typical Herpetomonas forms (Pl. 31, fig. 1). This form had a body 25 μ to 30 μ long, and the flagellum was 30 μ long. Its movements were characteristic, the body being clumsily swung from side to side by the lashing of the long thick flagellum. With Giemsa's stain the double character of the flagellum described by Prowazek is evident in most cases. If, as Patton (**17**) states, this is merely a stage in division, then it is an unusual type of division, since the kinetonucleus is not even transversely elongated when the flagellum has divided along its whole length, this being very unlike the state of affairs found by me in dividing forms (Pl. 31, fig. 9). My material for the study of these forms has been very scanty, and I can only say that they are very different, both in appearance and size, from the other flagellates met with in the fly.

(2) A commoner form (Pl. 31, figs. 2–14) was found in the intestine, and once in the Malpighian tubules. These infections were always heavy ones, the parasites occurring in dense clusters, either on the intestinal wall or free in the lumen of the intestine. On slides the clusters were seen to be formed by the typical rosettes, or more correctly, agglomerations, with the flagella pointing to the centre, as described by Woodcock (**26**) for cultural forms of trypanosomes. The body of this form was 15 μ to 18 μ long, and its movement was rapid and graceful, the anterior part of the body often undulating. A large number of dividing forms were usually present (Pl. 31, figs. 6–10). In one case, in which the forms were particularly elongated (Pl. 31, fig. 14), cysts were also found, and doubtful intermediate stumpy forms. The characters to which I wish to draw particular attention are the varying position of the kinetonucleus and the presence of an undulating membrane (Pl. 31, figs. 3, 4, and 11–14). (The forms shown in figs. 3, 4, and 11 are from the same fly). All intermediate stages between the short form, with its

anterior end truncated, and the elongated one with anterior extremity drawn out into a membrane attached to the flagellum, are found (Pl. 31, figs. 3, 4, and 11). A form resembling that in fig. 11 was found by Chatton and Alilaire (2) in Drosophila confusa, and described under the name Trypanosoma drosophilæ, although the authors recognised the possibility of its being a stage in the life-history of a Leptomonas found by them in the same fly. Werner (24) also described the same form from "Stubenfliegen," and named it Crithidia muscæ domesticæ to distinguish it from the biflagellate Herpetomonas muscæ domesticæ of Prowazek. Miss Mackinnon (14) also, whose paper appeared while this work was in progress, in describing what she regards as a Herpetomonas from Homalomyia corvina (?) pointed out the similarity between some of the forms found by her and the Crithidia of Werner, which would not be surprising if both are stages in the life-history of organisms belonging to the same genus, possibly to the same species. The forms shown in Pl. 31, figs. 12, 13, and 14 possess, undoubtedly, an undulating membrane, though the flagellum is not produced beyond it, and these resemble in a striking manner some stages of Trypanosoma cazalboui in cultures, described by Roubaud (22, pl. viii, figs. 2 and 6), thus indicating a close relationship between the parasite of a non-blood-sucking fly like Homalomyia and the trypanosomes of vertebrates. Patton (18, and 17, p. 142, note), in objecting to Prowazek's account of Herpetomonas (which, however, has been supported by other observers, Lingard and Jennings [12], Roubaud [22]) decided that all uni-flagellate parasites of insects with the kinetonucleus anterior to the trophonucleus and without undulating membrane are to be called Herpetomonas, and that those having the kinetonucleus posterior to the trophonucleus, and possessing an undulating membrane, should receive the generic name of Crithidia. Lühe (13) and Hartmann and Jollos (5) have pointed out that Patton's failure to see the characters observed by Prowazek and others does not prove their non-existence; and as to his

use of the name Crithidia, this is certainly a misuse of Leger's term, which he applied (**8**) to a short rounded form, "en form de grain d'orge légèrement aplati et tronqué a l'extrémité antérieure . . . ," and usually without an undulating membrane. However, it seems from the evidence of the forms found in Homalomyia that the same organism may be without an undulating membrane at one stage of its life-history, while possessing one at another stage. I shall return later in this paper to this question of nomenclature.

(3) In the rectum, near the rectal glands, were found masses of small oval bodies (Pl. 31, fig. 15) attached to the rectal epithelium. On examining these in water I was able to observe the mass apparently swell, as though the walls of the oval bodies were gelatinous, and after a short time some of the bodies were seen to become actively motile, with a small anterior flagellum (Pl. 31, figs. 16 and 17). The flies containing these cysts had no other flagellate stages in them, but came from the same locality as those that had. Similar cysts have been described by Minchin (**15**) for T. grayi, by Prowazek (**20**), Rosenbusch (**21**), and Mackinnon (**14**) for Herpetomonas, the latter having observed them giving rise to flagellates. The cysts stained with Giemsa (Pl. 31, fig. 15) show a faint trophonucleus and a distinct kinetonucleus, with a large number of scattered granules stained a deep purple colour, and have a definite wall surrounded by a remarkable substance which stains deeply, and may be gelatinous in nature (vide supra). But iron-hæmatoxylin shows little of these peculiar effects (Pl. 31, fig. 17A). The commencement of development of the flagellum is indicated by a clear area in Giemsa preparations (Pl. 31, fig. 15B), the borders of which appear to stain with iron-hæmatoxylin, showing a triangular area with the kinetonucleus as base (Pl. 31, fig. 17A and D), and the same appearance has been seen in non-flagellate forms of T. lewisi by Prof. Minchin, who kindly showed me his original drawings.

In the life-cycle of Leptomonas, as far as I have investigated it, we find the following forms: (1) A typical Lepto-

monas (fig. 2), which actively divides in the intestine or in the Malpighian tubules of the fly (figs. 6–10), producing (2) very active, slender forms, often with undulating membrane (figs. 11–14). These probably encyst while attached in large numbers to the rectal wall, and the cysts (figs. 15 and 17A) may be passed out with the fæces to give rise to flagellate forms in another fly, as described by Patton (19) for the Herpetomonas (? Leptomonas) of Musca nebulo, the Madras bazaar fly. But whether the large Herpetomonas form (fig. 1) should have a place in this life-history I am at present unable to decide. Almost certainly the above is but a part of the whole life-cycle, and the low percentage of infections have prevented the completion of it up to the present. It might be thought improbable on à priori grounds that flies in England and in India should be infected by the same pair of parasites, yet in smears of house-flies' guts which Dr. Row brought from India and kindly left at the Lister Institute, there are large Herpetomonads and small Leptomonads just as in H. canicularis in England. If these should prove to be different forms of the same organism, and at the same time have a trypanosome-stage in their life-history, considerable changes in our nomenclature of flagellate parasites will be necessitated.

As to Prowazek's description of elaborate autogamy and hereditary infection in Herpetomonas, one is tempted to interpret some of his figures (which hardly bear out his account), as being those of a Sporozoan infection, and I hope to publish shortly an account of a Microsporidian which I have found in Homalomyia.

The nomenclature of these forms, interesting on account of their probable relationship with the trypanosomes, is in a very confused state, and it is with a view to the clearing up of at least one part of the vexed question that I wish to re-state the following facts in their history.

Saville Kent in 1881 (23) established the genera Leptomonas and Herpetomonas for uniflagellate parasites found in a Nematode, Trilobus, and in Musca domestica respectively. The only points of distinction mentioned by him

which are of any service are that Leptomonas was $\frac{1}{2300}$ in. long, and formed rosettes, while Herpetomonas was $\frac{1}{650}$ to $\frac{1}{430}$ in. long, and had, at any rate, not been seen in rosettes or agglomerations. In 1902 Leger (**9**) found flagellate parasites in Homalomyia and other Diptera, and named an elongated form Herpetomonas (sp. var.), while a short rounded form, "en form de grain d'orge," he called Crithidia (sp. var.). Later (**10**, A and B), he described H. subulata from Tabanus as possessing an undulating membrane, still retaining the name Crithidia for short pyriform forms. Prowazek (**20**) in 1904 had investigated the parasite of the house-fly, and described it as possessing two flagella united by a membrane and arising from an anterior double basal-granule or diplosome. Novy, MacNeal, and Torrey, in 1907 (**16**) followed Leger's nomenclature for types found in mosquitoes, their Herpetomonas in cultures showing an undulating membrane. They described a diplosome, not where Prowazek had placed it, but at the posterior end of the body, and bearing, as they themselves point out, a considerable resemblance to a Diplococcus, which was generally adherent to the body of Herpetomonas in the cultures. Lingard and Jennings (**12**) in 1906 found in a Muscid fly forms showing the typical diplosome described by Prowazek, but most of their figures are not clear, and they claim to have seen the actual folding of the flagellate to form the biflagellate condition according to the Prowazek-Schaudinn theory respecting the origin of the double flagellum.

The history of Herpetomonas up to this point has been related in greater detail by Woodcock (**25**). His conclusions are—(1) That some of these parasites of mosquitoes are probably connected with Trypanosomes of vertebrates; (2) some of the typical Herpetomonads found may be simply and primarily parasites of the insects; (3) that forms adapted for life in sanguivorous insects, by which are meant "Crithidia" forms with an undulating membrane, following Patton's nomenclature, may be unrelated to any trypanosome

of a vertebrate. But no forms were then known with an undulating membrane in a truly non-sanguivorous insect. In 1908, however, Chatton and Alilaire (2) described flagellates found in Drosophila confusa—a Leptomonas (as distinct from Prowazek's Herpetomonas) and a Trypanosoma without a clear undulating membrane, but with the blepharoplast at the posterior end of the body. They named these forms L. drosophilæ and T. drosophilæ, but at the same time put forward the suggestion that they are really two stages of the same life-cycle. Werner (24) in 1909, and Rosenbusch (21) in 1910, have stated that there are two distinct parasites of the house-fly, a Herpetomonas of Prowazek and a Crithidia with posterior kinetonucleus, of which Rosenbusch describes the encystation. Roubaud, in an interesting article in 1909 (22), has used an old generic term, "Leptomonas," for the uniflagellate parasite of the fly Pycnogonum, excluding Herpetomonas of Prowazek, which he also found in the same fly. He regards, then, Herpetomonas of Prowazek as biflagellate, and Leptomonas as uniflagellate, with kinetonucleus usually anterior, but with a so-called trypanosome stage in its life-history. The evidence of Rosenbusch (21), Chatton and Alilaire (2), and Mackinnon (14), and that given by my figures, all goes to show that a form resembling Leptomonas of Saville Kent is found in non-sanguivorous flies (in three cases, house-flies), developing in the course of its life-history a form resembling a cultural trypanosome, and having an encysted stage. The fact that many observers have seen a large form (shown in Pl. 31, fig. 1), which differs very much in appearance from Leptomonas, renders it possible that the other observers who fail to see the two flagella are dealing with a different organism.

This much, however, seems certain: (1) That Leger's original pear-shaped Crithidia is only a stage of the Leptomonas life-history; also (2) that the "Crithidia" of later authors—Patton (18), Woodcock (25)—found in blood-sucking flies, or in cultures, are in some cases developmental stages of a Trypanosoma. The evidence of the forms found by me

(Pl. 31, figs. 11–14) in the house-fly, Homalomyia canicularis, shows that Rosenbusch's Crithidia muscæ domesticæ, and therefore probably Trypanosoma drosophilæ of Chatton and Alilaire, are merely forms assumed by a Leptomonas.

Should Leptomonas or Herpetomonas be the name given to these parasites of the Insecta? The Leptomonas of Saville Kent was described as being of a size comparable with that of the small Leptomonas, of, e.g., Homalomyia, whereas Herpetomonas was evidently a huge form. Again, Leptomonas was said to form rosettes. A diagnosis based on morphological grounds is of more value than one depending upon habitat. At present, therefore, Leptomonas would appear to be a correct name for the uniflagellate parasites found in the gut of non-sanguivorous insects, including house-flies, Pycnogonum (**22**), Bombyx (**11**), and in some plants (**7**), while Herpetomonas may be retained as a provisional name for a large form with peculiar flagellar apparatus and a complicated life-history, as described by Prowazek. Should the latter prove to be but a stage in the Leptomonas' life-history, then Herpetomonas should be merged in Leptomonas, since the latter would then have been the first which was accurately described. Crithidia cannot be applied as a generic name to any form, as it has simply been the name given to two stages in the life-history of Leptomonas, or in other cases to what are probably stages of Trypanosoma. That Leptomonas had priority over Crithidia was pointed out by Hartmann and Jollos (**5**), but it was not clear then that "Crithidia" was a form in the Leptomonas' life-history.

A paper by Flu on parasites of the house-fly, Musca domestica, appeared ('Centralblatt f. Bakt., etc.,' Bd. lvii, 1911, p. 522) after this paper had been sent to press, and is in the main confirmatory of the chief points emphasised above.

Protozoological Laboratory,
Lister Institute,
London.

References.

1. Chagas. — "Nova tripanozomiaze humana." 'Mem. do Inst. Oswaldo Cruz,' t. 2. f. 2. p. 159 (Abs. in 'Bull. Inst. Past.,' viii. p. 373).
2. Chatton et Alilaire.—"Coexistence d'un Leptomonas (Herpetomonas) et d'un Trypanosoma chez un Muscide non vulnerant. Drosophila confusa, Staeger," 'C. R. Soc. de Biol.,' t. 64, 1908, p. 1004.
3. Doflein.—'Lehrb. d. Prot.,' 2 Aufl., 1909.
4. Donovan.—'Kala Azar in Madras' (read before Bombay Med. Congr., February 24th, 1909.)
5. Hartmann and Jollos.—"Die Flagellatenordnung Binucleata," 'Arch. f. Prot.,' Bd. xix, 1910, Heft. 1.
6. Hewitt.—"The Bionomics, Allies, Parasites, and the Relations of M. domestica to Human Disease," 'Quart. Journ. Micr. Sci.,' 54, 1909, p. 347.
7. Lafont.—"Sur la presence d'un Leptomonas dans trois Euphorbiacées," 'Ann. de l'Inst. Past.,' xxiv. 1910. p. 205.
8. Léger.—"Sur un flagellé parasite de l'Anopheles maculipennis," 'C. R. Soc. de Biol.,' liv. 1902, p. 354.
9. ———"Sur quelques Cercomonadines nouvelles ou peu connues parasites de l'intestine des Insectes," 'Arch. f. Prot.,' Bd. ii, 1902, p. 180.

10A. ——— "Sur un nouveau Flagellé parasite des Tabanides." 'C. R. Soc. de Biol.,' t. 57, p. 613.

10B. ——— "Sur les affinités de l'Herpetomonas subulata, et la phylogenie des Trypanosomes," 'C R. Soc. de Biol.' t. 57, p. 615.

11. Levaditi.—"Sur un nouveau Flagellé parasite du Bombyx mori." 'C. R. Acad. des Sciences,' cxli. 1905. p. 631.
12. Lingard and Jennings.—'Some Flagellate Forms found in Diptera,' Adlard and Son, London, 1906.
13. Lühe.—"Die im Blute schmarotzenden Protozoen und ihre nächsten Verwandten," Mense's 'Handbuch der Tropenkrakh., 1906.
14. Mackinnon. — "Herpetomonads from the Alimentary Tract of certain Dung-flies," 'Parasitology," iii. September, 1910, p. 255.
15. Minchin.—"Investigations on the Development of Trypanosomes in Tsetse-flies, etc.," 'Quart. Journ. Micr. Sci.,' 52, p. 159.

16. Novy, MacNeal and Torrey.—"The Trypanosomes of Mosquitoes and other Insects," 'Journ. Infect. Diseases,' iv, No. 2, p. 223.

17. Patton.—"The Life cycle of a Species of Crithidia parasitic in Gerris fossarum," 'Arch. f. Prot.,' xii, 1908, p. 131.

18. ——— "A Critical Review of our Present Knowledge of the Hæmoflagellates and Allied Forms." 'Parasitology,' ii, May, 1909, p. 91.

19. ——— "Experimental Infection of the Madras Bazaar fly, Musca nebulo, with Herpetomonas muscæ domesticæ," 'Bull. Soc. Patholog. Exot.,' iii, p. 264.

20. Prowazek.—"Die Entwickelung von Herpetomonas," 'Arb. a. d. Kais. Ges.,' xx, 1904, p. 440.

21. Rosenbusch.—"Eine neue Encystierung bei Crithidia muscæ domesticæ," 'Centr. f. Bakt.,' Bd. lv, 1910, p. 387.

22. Roubaud.—"Les Trypanosomes pathogenes et la Glossina palpalis," 'La Maladie du Sommeil au Congo Français,' 1909.

23. Saville Kent.—'Manual of the Infusoria,' 1880–81.

24. Werner.—"Über eine eingeisselige Flagellatenform im Darm der Stubenfliege," 'Arch. f. Prot.,' xiii, 1909.

25. Woodcock.—"The Hæmoflagellates and Allied Forms," in Lankester's 'Treatise on Zoology,' vol. i, fasc. i, 1909, p. 193.

26. ——— "Studies on Avian Hæmoprotozoa," 'Quart. Journ. Micr. Sci.,' 55, 1910, p. 641.

EXPLANATION OF PLATE 31,

Illustrating Mr. J. S. Dunkerly's paper "On Some Stages in the Life-history of Leptomonas muscæ domesticæ, with Some Remarks on the Relationships of the Flagellate Parasites of Insects."

[All figures are outlined with the aid of Zeiss-Abbé drawing apparatus, and are drawn at a magnification of 2400.]

Fig. 1.—Large Herpetomonas from stomach of Homalomyia canicularis. Osmic vapour, Giemsa.

Fig. 2.—Leptomonas from intestine of H. canicularis, showing distinct blepharoplast. Flemm.-Fe. hæm.

Figs. 3 and 4.—Leptomonas from intestine of H. canicularis, showing varying positions of the kinetonucleus. Schaud-Fe. hæm.

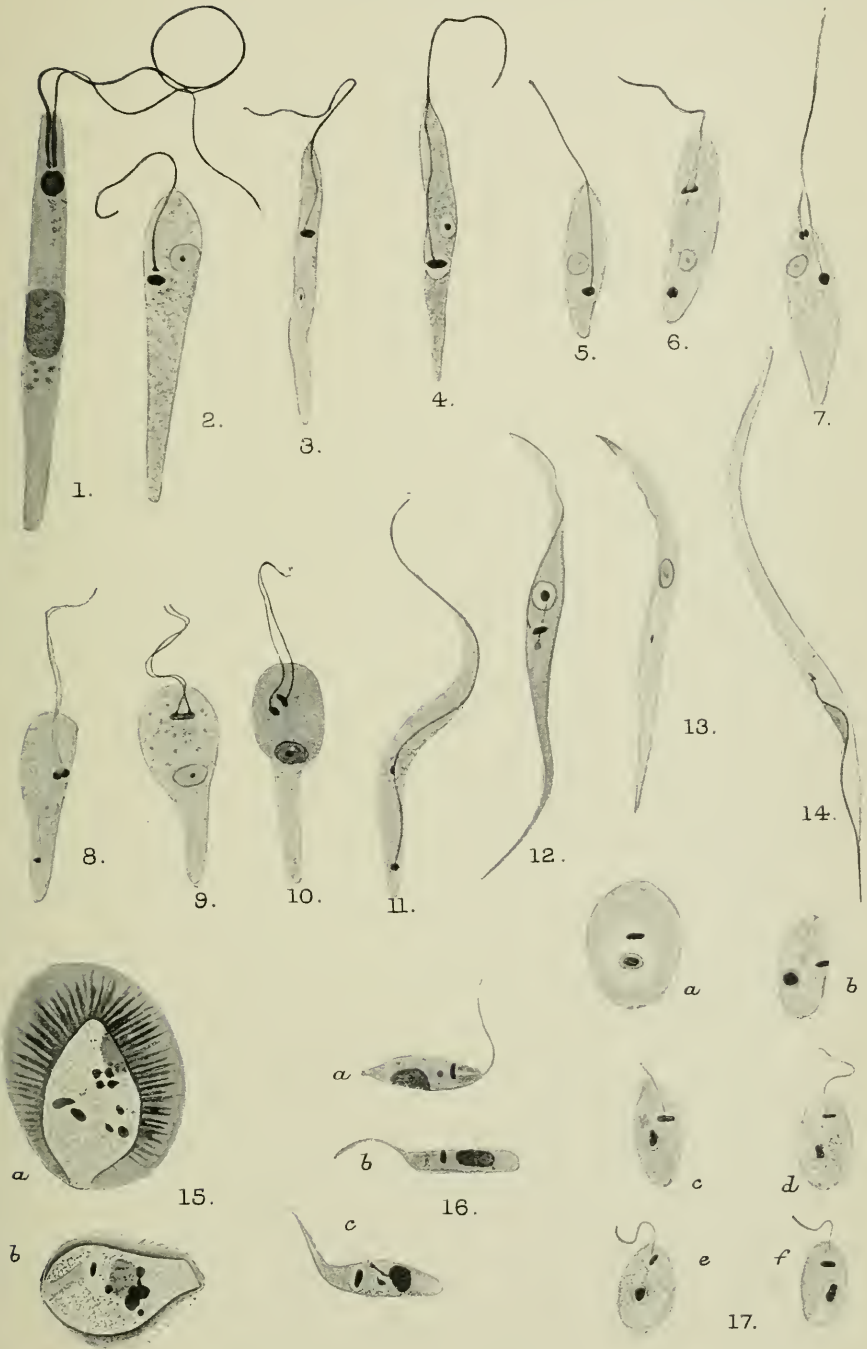
Figs. 5–10.—Leptomonas from intestine of H. canicularis; various stages in division. Flemm.-Fe. hæm.

Figs. 11–14.—Leptomonas from intestine of H. canicularis; trypaniform individuals, Schaud.-Fe. hæm.

Fig. 15.—Cysts of Leptomonas muscæ domesticæ from rectum of H. canicularis, showing scattered nuclear material. Osmic vapour, Giemsa.

Fig. 16.—Small flagellate forms a few minutes after leaving cyst. Osmic vapour, Giemsa.

Fig. 17.—Small flagellate forms a few minutes after leaving cyst showing development of the flagellum. Flemm.-Fe. hæm.



LEPTOMONAS MUSCAE DOMESTICAE.

Abdruck aus dem
Centralblatt f. Bakteriologie, Parasitenkunde u. Infektionskrankheiten.
I. Abteilung. Originale.
Herausgeg. von Prof. Dr. **O. Uhlworm** in **Berlin.** — Verlag von **Gust. Fischer** in **Jena.**
62. Bd. 1912. Heft 1/2.

On the occurrence of Thelohania and Prowazekia in Anthomyid flies.

[Protozoological Laboratory, Lister Institute, London.]

By **J. S. Dunkerly,** B. Sc., London.

With 1 Plate.

I. Thelohania ovata.

In searching flies for flagellate parasites, I found in one an infection of small spores in groups, and resembling superficially yeast-like bodies which are sometimes present in flies' intestines. The fly, Homalomyia scalaris, was not one of those which I was specially examining, but was an odd capture, and for that reason I did not pay much attention to it at the time. A smear of the teased-up rectum was fixed in osmic vapour and stained with Giemsa, but it was not until some time afterwards that I examined the slide, when it was seen that the spores were those of a Microsporidian, and I have to thank Dr. Woodcock for his kind assistance in directing me to the literature on the subject.

Isolated meronts were found, containing one, two, four or more nuclei (figs. 1—5), and some of these are apparently budding off uninucleate bodies (fig. 2), which may become either meronts or possibly sporonts. Exactly similar bodies are described by Perrin (8) for Pleistophora periplanetae and by Stempell (11 and 12) for Pleistophora (Thelohania) mülleri, while Shiwago (14) states that young pansporoblasts (sporonts) in Pl. periplanetae may bud in the same way. The sporonts each form eight spores (sporoplasts) which can be seen in various stages of development (figs. 6 and 7), but the early divisions are not clear in this material. Each spore has at first an almost colourless cytoplasm and a mass of material at either end which stains red with Giemsa. It is sen early however, that one of these masses is purplish red, while the other is a brighter red colour (fig. 6). It is unfortunate that only Giemsa-stained material was available, as probably the spore is developed from a pansporablast as is well described for Thelohania giardi by Mercier (6); but during certain stages in the development of spores in Th. chaetogastris

the nuclei are terminal according to Schröder (10), and probably the bright red nuclear material (fig. 7) forms the „Amöboidkerne" and the pink vacuole is the polar capsule. The larger spores are about 6 μ to 7 μ long and are more elliptical than ovoid in shape, but besides these there are a few groups of small spores (fig. 8), which are about 4 μ long, and may represent microspores, in which case the larger ones must be termed macrospores. Perrin, who worked with Pl. periplanetae (8) described two kinds of trophozoite and spores in Pleistophora, but thought that the smaller forms belonged to an undescribed species, but microspores and macrospores have been described in several Microsporidia; in Pleistophora mirandellae by Vaney and Conte (15), in Pl. elegans by Auerbach (1a), in Thelohania janus and in Gurleya legeri by Hesse (3 and 4), in Th. chaetogastris by Schröder (10), and in Glugea varians by Leger (5). It seems likely therefore that the two kinds of spores found (figs. 7 and 8) represent macrospores and microspores of the same organism.

The material allows of no more than a record of the occurrence of this Microsporidian, which I name Thelohania ovata in an Anthomyid fly. Besides the Thelohania found by Hesse (3) in Tanypus, species of Glugea have been described also as parasitic in Diptera; e. g., in Simulium ornatum larva by Leger (5). Vosseler in 1897 (16) described what may have been the trophozoite stage of a Microsporidian infecting Musca (Calliphora) vomitoria and Sarcophaga carnaria with fatal results, but apparently he did not see the actual spores.

Flu (2) has published a description of a parasite in houseflies which seems to resemble in many of its stages the organism described above. The spores, of which eight are formed in a cyst, do not appear to possess a polar capsule, and Flu classes the organism discovered by him as a Schizogregarine, naming it Octosporea muscae domesticae. A point of considerable theoretical interest is the rather striking resemblance which the trophozoites, especially when budding (figs. 2 and 3), bear to Prowazek's figures (9, fig. 7 *j*) of parthenogenesis in Herpetomonas muscae domesticae, and Flu has pointed out that the same may be said of stages in his Octosporea. It certainly seems probable that stages of some Sporozoan parasite have been included by Prowazek in the life-history of Herpetomonas.

Chatton and Krempf have recently[1]) described two parasites from Drosophila confusa, which they identify with Octosporea Flu, one with eight spores, O. muscae domesticae Flu, and one with a single spore in sporont, O. monospora Chatton and Krempf. They object to the classification of Microsporidia based on the number of spores in each sporont, owing to the variability of this character, but on their own showing, nothing is to be gained by founding a genus Octosporea with no character of distinction from Thelohania. I have retained therefore the provisionally effective generic name of Thelohania for this Microsporidian with sporont containing eight spores each with one polar capsule.

1) Bull. de la Soc. Zool. de France. T. 36. 1911. p. 172—179. Text fig.

II. Prowazekia sp.

In one fly, Homalomyia canicularis, which I examined for Leptomonas, the rectum contained numerous flagellates resembling Bodo, with one anterior and one posterior flagellum. Smears were made and stained with Giemsa after osmic vapour, and iron haematoxylin after Schaudinn's sublimate alcohol, and in examination showed, that the Bodo possessed a large deeply staining body situate near the base of the flagella (figs. 9—13). With Giemsa the large nucleus stained distinctly red, while the elongated body at the base of the flagella takes on a very dark lilac or purple colour. Besides these bodies, chromatic granules are constantly present, and vacuoles are seen in some cases. The specimens stained with iron haematoxylin similarly show a very clear vesicular nucleus with a large karyosome, a darkly staining elongated body at the base of the flagella and numerous irregular staining granules. The length of the iron haematoxylin specimens is about 6 μ, but those fixed with osmic vapour and stained with Giemsa are larger and seem to be flattened out. No clear division stages are to be found, and the basal granules of the flagella are not obvious.

It would seem that this organism is a form of Prowazekia discovered by Hartmann and Chagas (4) in a culture of human faeces in Brazil, and also found free-living by Nägler (7), and in the human intestine by Mathis and Leger (6). Alexeieff (1) has objected to the creation of a new genus, asserting that Prowazekia is really Bodo, and that the chromatic mass at the base of, the flagella is not nuclear in structure or behaviour, and at the same time he says of Hartmann's group, the Binucleata. "C'est un groupement purement théorique et Hartmann a tort de vouloir l'introduire en systématique." In a later paper (2) however, he describes the behaviour of the body at the base of the flagellum, in an organism identified by him as Bodo caudatus, at the time of division as resembling a nucleus in process of division and this view must be taken as modifying his previous statements regarding the non-nuclear character of this body in Prowazekia, although he himself persists in regarding Prowazekia as a nomen nudum. A typical Bodo, according to Prowazek (9), may have such a body, but he did not consider it nuclear in character, naming it simply „Geißelsäckchen". At present therefore, the distinction between Bodo and Prowazekia is somewhat uncertain in character, and it is possible that many organisms previously described as Bodo will ultimately prove to be Prowazekia.

The rarity of these two parasites of flies described above seems to point to a casual infection, due to the well known propensity of flies to settle on any decomposing material, and it may be as well to remember in this connection that Microsporidia found in Stegomyia were stated to be the connected with yellow fever, though this has been denied by later workers, while at least two species of Prowazekia are found in human faeces.

September, 1911.

Reference.

I. Thelohania.

1) Auerbach, Die Cnidosporidien. Leipzig 1910. (Contains a valuable bibliography to which I am much indebted.)
1a) —, Zwei neue Cnidosporidien aus cyprinoiden Fischen. (Zool. Anz. Bd. 36. 1910. p. 440.)
2) Flu, Studien über die im Darm der Stubenfliege Musca domestica vorkommenden protozoären Gebilde. (Centralbl. f. Bakteriol. Abt. I. Orig. Bd. 57 1911. p. 522.)
3) Hesse, Sur la présence de Microsporidies du genre Thelohania chez les Insectes. (Compt. Rend. Acad. Scienc. T. 137. 1903. p. 418.)
4) —, Sur une nouvelle microsporidie tétrasporée du genre Gurleya. (Compt. Rend. Soc. Biol. T. 55. 1903. p. 495.)
5) Leger, Sur une nouvelle Myxosporidie de la famille des Glugéidées. (Compt. Rend. Acad. Scienc. T. 125. 1897. p. 260.)
6) Mercier, Sur la développement et la structure des spores de Thelohania giardi. (Compt. Rend. Acad. Scienc. T. 146. 1908. p. 33.)
7) Minchin, Sporozoa. (Lankesters Treatise on Zoology. London 1903.)
8) Perrin, Observations on the structure and life-history of Pleistophora periplanetae. (Quarterly Journ. Microsc. Science. Vol. 49. 1906. [Preliminary note.] (Proc. Cambridge Phil. Soc. Vol. 13. p. 204.)
9) Prowazek, Die Entwickelung von Herpetomonas. (Arbeit. a. d. Kaiserl. Gesundheitsamt. Bd. 20. p. 440.)
10) Schröder, Thelohania chaetogastris, eine neue in Chaetogaster diaphanus schmarotzende Mikrosporidienart. (Arch. f. Protistenk. Bd. 14. p. 119.)
11) Stempell, Zur Entwickelung von Pleistophora Mülleri. (Zool. Anz. Bd. 24. 1901. p. 157.)
12) —, Ueber Thelohania Mülleri. (Zool. Jahrb. Abt. f. Anat. Bd. 16. 1902. p. 235.)
13) —, Zur Morphologie der Mikrosporidien. (Zool. Anz. Bd. 35. 1910. p. 801.)
14) Shiwago, Ueber Vermehrung bei Pleistophora periplanetae. (Zool. Anz. Bd. 34. 1909. p. 647.)
15) Vaney and Conte, Sur une nouvelle Microsporidie, Pleistophora mirandellae. (Compt. Rend. Acad. Scienc. T. 133. 1901.)
16) Vosseler, Ueber eine seltsame Infektionskrankheit bei Fliegen. (Jahresber. d. Ver. vaterl. Naturk. in Württemberg. Bd. 53. 1897. p. 242.)

II. Prowazekia.

1) Alexeieff, Sur quelques points de la structure des "Binucléates" de Hartmann. (Compt. Rend. Soc. Biol. T. 69. 1910. p. 532.)
2) —, Sur la morphologie et la division de Boda caudatus (Duj.) Stein. (Compt. Rend. Soc. Biol. T. 70. 1911. p. 130.)
3) —, Sur les Flagellés intestinaux des poissons marins. (Arch. Zool. expér. T. 6. 1910. p. 1.)
4) Hartmann u. Chagas, Flagellaten-Studien. (Mem. d. Inst. Oswaldo Cruz. T. 2. 1910. p. 64.)
5) Hartmann u. Jollos, Die Flagellatenordnung Binucleata. (Arch. f. Protistenk. Bd. 19. 1910. p. 81.)
6) Mathis et Leger, Sur un Flagellé, Prowazekia Weinbergi n. sp. fréquemment observé dans les selles de l'Homme. (Bull. Soc. méd. chir. de l'Indo-Chine. T. 1. 9 oct. 1910 [Abs. in Bull. Inst. Past. T. 9. 1911. p. 198].)
7) Nägler, Prowazekia parva n. sp. (Arch. f. Protistenk. Bd. 21. 1910. p. 111.)
8) Parker, Beyer and Pothier, A study of the etiology of yellow fever. (Yellow Fever Inst. No. 1. Bull. No. 13. March 1903.)
9) Prowazek, Flagellatenstudien. (Arch. f. Protistenk. Bd. 2. 1903. p. 195.)

Explanation of figures.

All figures were outlined with the aid of Abbes Drawing Apparatus.

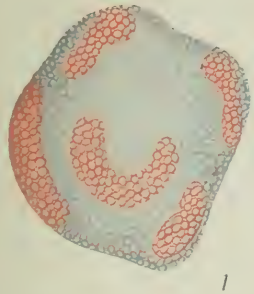
I. Thelohania ovata.

Fig. 1. Trophozoite (meront) with five nuclear masses. ×2000.
Fig. 2. „ „ budding off uni-nucleate bodies. ×2000.
Fig. 3. „ „ „ „ „ „ ×1250.
Fig. 4. Binucleate meront, with nuclei again dividing. ×2000.

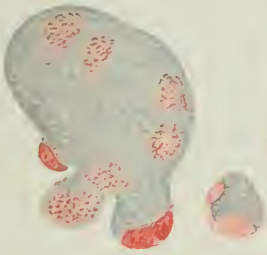
Fig. 5. Meront or (?) sporont, with four nuclei. × 2000.
Fig. 6. Eight developing sporoplasts, scattered owing to bursting of sporont wall. × 1000.
Fig. 7. Eight macrospores in sporont. × 1250.
Fig. 8. Group of microspores. × 1000.

II. Prowazekia sp.

Fig. 9. Prowazekia sp. — Osmic vapour-Giemsa. × 2000.
Fig. 10. „ „ „ „ „ × 2000.
Fig. 11. „ „ „ „ „ × 2000.
Fig. 12. „ „ Corr.-alc.-Iron haem. × 2000.
Fig. 13. „ „ „ „ „ × 2000.

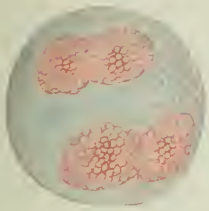


1

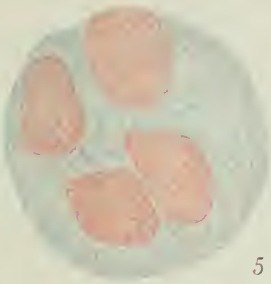


2

3

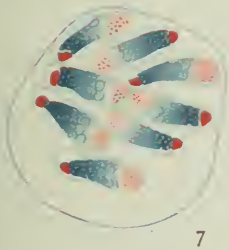


4



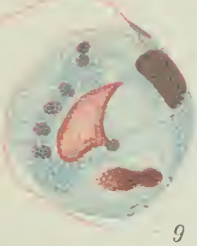
5

6

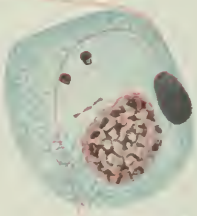


7

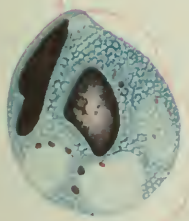
8



9

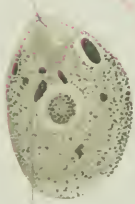


10



11

12

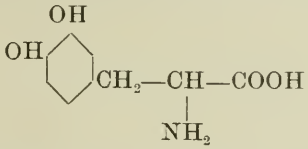


13

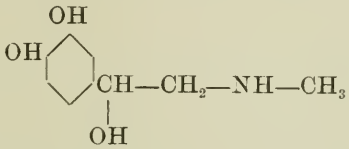
Journal Physiology, Vol. XLIII.
[*From the Proceedings of the Physiological Society*, July 22, 1911.]

Note on the probable formation of adrenaline in the animal body. By Casimir Funk.

Some months ago the author prepared the 3–4 dihydroxyphenylalanine



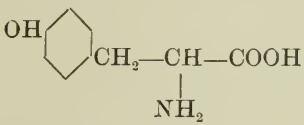
(which is probably present in small quantities in proteins) with the intention of ascertaining whether it might possibly be the precursor of adrenaline



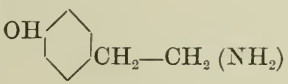
Experiments showed that the compound had practically no action on the blood-pressure and was without any marked toxic power when injected. As many other catechol derivatives show very marked toxic and vaso-constricting actions, it appears probable that the presence of the side chain causes this difference in action.

Experiments were carried out to ascertain if adrenaline could be obtained from the 3–4. dihydroxyphenylalanine by incubating with suprarenal glands, but gave negative results. A negative result was also obtained when the compound was incubated with a mixture of suprarenal glands, liver and pancreas, it being thought that possibly the transformation to adrenaline might take place in several steps in different organs.

The experiments do not prove that this substance is not a precursor of adrenaline, since the first stage in the formation of the latter may take place in the intestine, and may be of a similar nature to the transformation in the intestine of tyrosine



into p-hydroxyphenylethylamine



[*Reprinted from the Journal of Physiology,*
Vol. XLIII. No. 5, *December* 22, 1911.]

ON THE CHEMICAL NATURE OF THE SUBSTANCE WHICH CURES POLYNEURITIS IN BIRDS INDUCED BY A DIET OF POLISHED RICE.

By CASIMIR FUNK.

(*From the Bio-chemical Department, Lister Institute of Preventive Medicine.*)

As a result of the work of a number of observers (Eykman(1), Gryns(2), and Fraser and Stanton(3)) it has been shown that the cortical layers of rice contain a substance which cures beri-beri in man and the polyneuritis which is produced in birds by feeding them on polished rice.

The present inquiry is directed to determine the chemical nature of the curative substance.

The experiments were carried out on pigeons of which a large stock was kept. Polyneuritis was induced by feeding on ordinary polished rice. The average time before the onset of symptoms was three weeks. The presence or absence of the active principle in the different fractions, obtained during the investigation of rice-polishings, was determined by administering them to pigeons already severely affected with polyneuritis and observing the result. The condition of the birds at the time of the tests was such that untreated they succumbed within 12 hours.

It may here be mentioned that the extracts, if given in too large a dose, were poisonous. This, as will be shown later, was due to the presence of choline which accompanied the active principle until the final stage of the separation. The poisonous action can be avoided by giving a dose calculated on the quantity of polishings used. With suitable doses passed into the crop by a tube, the pigeons recovered in 6–12 hours. Often, even after three hours the bird seemed quite well.

One symptom of polyneuritis in pigeons is paralysis of the crop and the birds being also generally paralysed the dose administered is very likely to run out again unless the head is supported. A further possible source of error occurs when the crop is not completely paralysed as the bird can vomit the material.

In more chronic cases of polyneuritis although recovery takes place and the animal feeds normally and appears well some amount of lameness persists due to degeneration of nerves. The pigeons which recovered after having received one dose of active substance were again fed on polished rice. Under these circumstances they showed symptoms of the disease for the second time in from 3 to 12 days according to the dose administered,

Extraction of rice-polishings. The method of extraction adopted was a modification of that used by Fraser and Stanton and gave a better yield than is obtained by their procedure. Since the amount of curative substance present in the rice-polishings is really very small, a quantity of substance amounting to 54 kilograms was used. This was exhausted in separate portions of $1\frac{1}{2}$ kilograms each with 4 litres of alcohol containing gaseous hydrochloric acid to the extent of 2–5 %. The extraction was hastened by the use of the shaking machine and the liquid afterwards filtered by means of a large Buchner funnel. The residue was pressed out in a hydraulic press and the liquid obtained added to the original filtrate. The yield of alcoholic extract obtained in this way was $3\frac{1}{2}$ litres for each portion treated.

Investigation of alcoholic extract. The alcoholic extract was evaporated in vacuo at 30° C. and 347 grammes of a fat-like substance obtained from each $3\frac{1}{2}$ litres. This residue was melted at 50° C. and heated with 1 litre of water on the water bath; it was then filtered at a temperature of 38° C.—40° C. The filtrate obtained formed two layers which were separated by decantation, and the aqueous layer extracted three times with ether in order to remove all fatty substances. This aqueous solution contained no proteins and gave none of the known reactions for amino-acids (Millon, glyoxylic acid, bromine, xanthoproteic diazo- and diacetyl-reaction).

When tested on pigeons suffering from polyneuritis it was found to be effective in doses corresponding to about 20 grammes of the original polishings.

Treatment of fraction soluble in water with phosphotungstic acid. The total aqueous extracts were now combined and amounted to 17 litres. After adding sufficient H_2SO_4 to give a 5 % solution the acid liquid was treated with a 5 % phosphotungstic acid solution and left to stand overnight. The precipitate obtained weighed when dry 900 grammes. The filtrate freed from phosphotungstic acid by means of $Ba(OH)_2$ was tested and found ineffective; it still contained traces of nitrogen. The phosphotungstic acid precipitate was washed with 5 % H_2SO_4, then ground up with $Ba(OH)_2$ in a mortar and after the addition of H_2O shaken for three hours. The barium phosphotungstate precipitate

was filtered off and washed thoroughly with water. Air was passed through the alkaline filtrate to get rid of ammonia and the excess of $Ba(OH)_2$ carefully precipitated by means of H_2SO_4 and separated. The filtrate which was still very alkaline with a strong methylamine smell, was quickly neutralised with HCl and evaporated in vacuo at ordinary temperature. On extracting the residue with absolute alcohol a considerable portion remained behind; this was found to consist mostly of inorganic chlorides and amounted to 21 grms. The solution thus obtained was freed from alcohol and was tested on six fowls and found to be effective in doses corresponding to about 40 grms. of the original rice-polishings. The solution was free from proteins, phosphorus and carbohydrates.

Treatment with mercuric chloride. In the next stage of purification mercuric chloride was used. The alcoholic solution described above was treated with an alcoholic solution of mercuric chloride and after standing in the cold for some time the *crystalline* precipitate obtained was filtered off and washed with cold alcohol. The weight of this precipitate was about 45 grms. and by partially evaporating the alcoholic solution 5 grms. more were obtained. The 50 grms. of mercury salt was recrystallised from water containing some mercuric chloride and 42 grms. of needle-like crystals obtained. Three portions composed of: (1) Crystalline substance, (2) Aqueous filtrate, (3) Alcoholic filtrate, were therefore obtained in treating with mercuric chloride as indicated in the following table:

Alcoholic solution of extract treated with mercuric chloride = alcoholic filtrate (3); ppt. of crystals which on recrystallising from H_2O gave = H_2O filtrate (2); crystalline substance (1)

These three portions were now investigated separately.

(1) *Treatment of the crystalline substance.* This substance was suspended in H_2O and decomposed with H_2S. The Hg-free liquid proved effective in curing pigeons when administered in doses equivalent to about 100 grms. of the original polishings.

As some preliminary experiments showed that the bulk of the above crystals consisted of choline, an aqueous solution was made and treated with $AgNO_3$ in the presence of $Ba(OH)_2$. This reagent does not precipitate choline but a small precipitate was obtained which was decomposed by H_2S and tested on one pigeon with positive result. The filtrate of the $AgNO_3$ and $Ba(OH)_2$ precipitate was proved to be without effect.

(2) *Treatment of mercuric chloride aqueous filtrate.* This filtrate was decomposed by H_2S, the precipitate filtered off and the liquid

evaporated in vacuo. The residue was dissolved in alcohol and fractionated by consecutive additions of an alcoholic platinic chloride solution. In this way eight fractions of a platinum chloride salt were obtained. All these were devoid of curative properties and were found on analysis to consist of choline.

·1718 grm. substance gave ·0544 Pt.

·2310 grm. „ required 7·5 c.c. N/10 H_2SO_4 (Kjeldahl),

i.e. 31·66 % Pt and 4·54 % N.

$(C_5H_4NOCl)_2 . PtCl_4$ requires 31·65 % Pt and 4·54 % N.

The alcoholic filtrate from the last platinum chloride precipitate was evaporated in vacuo, the residue dissolved in H_2O and the platinum eliminated by H_2S. This solution was given to two pigeons and found effective in doses corresponding to 40 grms. of polishings.

As the platinic chloride salt of choline is known to be very insoluble and the choline itself was proved to be ineffective, it was thought that another base must be present in the filtrate. Mercuric chloride was again added but in this case no insoluble salt was formed; it would seem that the active substance is but partially precipitated by mercuric chloride and then only in the presence of some other substances such as choline. An attempt to form a picrate was equally unsuccessful. These points suggested that the curative substance was probably present in all the mercuric chloride fractions and curing experiments proved this surmise to be correct.

Since it was difficult to find a suitable precipitant for the active substance phosphotungstic acid was again used. By this means 1·4 grms. of a crystalline phosphotungstate were obtained. This salt, which was entirely insoluble in H_2O, was recrystallised from dilute alcohol; in this way 1·1 grms. of a crystalline substance composed of five and six sided prisms were obtained. This substance gets black when heated to 200° C. and does not melt at 300° C. It contains 1·59 % of nitrogen after drying in vacuo at 110° C. Half a gram of this substance was decomposed with baryta and the excess of barium removed by CO_2.

On administering the filtrate to two pigeons suffering from polyneuritis they quickly recovered. Since each dose contained only 4 mgrs. of nitrogen it is evident that a very small amount of the substance is effective.

As the amount of this substance obtained was insufficient for further analyses it is at present impossible to say anything with regard to its chemical nature.

(3) *Treatment of mercuric chloride alcoholic filtrate.* This filtrate was evaporated in vacuo and the residue extracted with water. On removing the Hg by means of H_2S a fluid was obtained which was

effective in curing pigeons. Chlorine was removed from the liquid by Ag_2SO_4, the Ag eliminated by H_2S and the H_2SO_4 by means of $Ba(OH)_2$.

To the alkaline solution was added $AgNO_3$ and $Ba(OH)_2$ as long as a precipitate continued to form. The precipitate, which was at first white but on standing became brown, was washed with water and decomposed by H_2S. A very dilute solution of H_2SO_4 was used to remove the last traces of barium and the filtrate was then tested on eight pigeons and found effective. The $AgNO_3 + Ba(OH)_2$ filtrate was freed from silver by hydrochloric acid and from barium by sulphuric acid; on testing it was found inactive.

The solution of the decomposed Ag salt was evaporated in vacuo and an endeavour made to crystallise the residue from dilute alcohol, but no crystals formed at first though some were obtained after the solution had been kept in a vacuum desiccator for some time. These crystals which consisted of long microscopical needles proved by their reactions with brucine and diphenylamine to be the nitrate of a base. They gave no reaction for substances such as Arginine, Histidine, Carnosine, and Creatinine which from their chemical behaviour might be present in this fraction. They left no residue on burning and melted at 233° C. This high melting point does not correspond with any of the nitrates of the known bases which might be present in this fraction. The product of the first crystallisation weighed 0·25 grm. and the second 0·15 grm. The crystals contained nitrogen and were free from Ba, Ag, Cl, and H_2SO_4. They were insoluble in cold water and in alcohol but dissolved with difficulty in hot water. They gave the following results on analysis[1] after being dried at 110° in vacuo.

·1481 grm. substance gave ·3021 grm. CO_2 and ·0706 grm. H_2O.
·0878 grm. „ „ 5·9 c.c. N (moist) at 749 m.m. and 16°.

Found 55·63 % C.
„ 5·29 % H.
„ 7·68 % N.

These figures correspond best to the formula $C_{17}H_{18}O_4N$ (HNO_3) for which following figures are required:

56·16 % C.
5·28 % H.
7·71 % N.

[1] By the time my work had reached this stage my material was nearly exhausted and only just enough for one analysis of carbon and nitrogen was available. I therefore asked my friend Dr Nierenstein, Lecturer in Biochemistry in Bristol, who at the present time is in constant practice in organic analyses, to undertake the determinations for me. Dr Nierenstein has been good enough to do this and I beg to express here my gratitude for his kindness.

Although the active substance has been definitely identified with the Ag salt obtained by precipitating with $AgNO_3$ in presence of baryta, the proof of activity of the nitrate obtained from this Ag salt and which was the salt submitted to analysis is less conclusive, as by this time the exhaustion of the material permitted of but four experiments, three of which were successful and one failed to cure the bird. For the same reason it was not possible to investigate further the chemical reaction of the substance but attempts to obtain by extraction of more raw material the active substance from other food-stuffs are in progress.

In conclusion I wish to express my thanks to Dr Leonard Braddon of Seremban (F.M.S.) for kindly supplying the necessary rice-polishings.

Summary.

(1) Polyneuritis of birds as shown by Eykman, Gryns, Fraser and Stanton, is due to the lack of an essential substance in the diet. The substance is only present in minute amount, probably not more than ·1 grm. per kilo of rice.

(2) The substance which is absent in polished rice and is contained in rice-polishings is an organic base which is completely precipitated by phosphotungstic acid and by silver nitrate and baryta. It is partially precipitated by mercury chloride in alcoholic solution in the presence of choline and is not precipitated by platinum chloride in alcoholic solution.

Reasons for provisionally regarding the active substance as a body giving a crystalline nitrate which has the percentage composition of 55·63 % C, 5·29 % H and 7·68 % N are adduced, but as by the time the search had approached the final stages the material became exhausted; duplicate analyses could not be made and but few animal experiments performed.

The chemical nature of the curative substance could not be further investigated immediately but larger quantities of raw material are being worked up.

(3) The curative dose of the active substance is small; a quantity of substance which contains 4 mgr. of nitrogen cured pigeons.

REFERENCES.

1. Eykman. Virchow's Arch. 148, 523, 1892; Ibid. 149, 187, 1897. Arch. f. Hygiene, LVIII. 150 (1906).
2. Gryns quoted by Schaumann. Arch. f. Schiffs- und Tropenhygiene, 1910.
3. Fraser and Stanton. Studies from the Institute for Medical Research. Federated Malay States, No. 12. The Etiology of Beri-beri, 1911.

[*Reprinted from the Journal of Physiology,*
Vol. XLIV. Nos. 1 *&* 2, *March* 29, 1912.]

THE EFFECT OF A DIET OF POLISHED RICE ON THE NITROGEN AND PHOSPHORUS OF THE BRAIN.

By CASIMIR FUNK.

(*From the Bio-chemical Department, Lister Institute of Preventive Medicine.*)

In my previous paper(1) I described the method for isolation from rice-polishings of a substance which cured polyneuritis in birds. The basic character of this substance and its occurrence in that fraction soluble in alcohol suggested the idea that the substance might be a constituent base of a lipoid or cerebroside(2). The basic constituents of lipoids have received little attention hitherto, but MacLean(3) has found that in certain lipoids about 60 % of the total nitrogen belongs to a base other than choline. From the severity of nervous symptoms in beri-beri and the increased secretion of nitrogen and phosphorus in the urine of beri-beri patients it seems probable that the symptoms may be due to a destruction of lipoids in the body, especially in the nervous tissues.

As a preliminary to investigating normal brains for the presence of such a base (this work being now in progress), experiments were made to ascertain whether any differences could be detected between the contents of nitrogen and phosphorus in the brains of normal pigeons and in those suffering from polyneuritis. Similar investigations have been carried out on the phosphorus content of brains of pigeons suffering from polyneuritis by Schaumann(4) who employed a slightly different method, but he was unable to find any differences chemically from normal brains.

Another point of interest was to determine the total nitrogen content of the brain in polyneuritis pigeons and to find the relationship between this and the nitrogen contained in a dose of the substance sufficient to cure. In previous experiments I have found that a very small dose (4 mgr.) of the basic substance was sufficient to cure. Schaumann(5) in a recent discussion on this subject suggested that the

substance therefore may act as a hormone. The average nitrogen content of a pigeon's brain suffering from polyneuritis was found to be about 33 mgr. and as the substance probably acts on the nervous tissue, the relation between the nitrogen content of the curative dose and the total nitrogen content of the brain appears too large to agree with Schaumann's hormone theory.

The brain of normal pigeons was found to contain on the average 9·77 % nitrogen and 1·84 % phosphorus and that of birds suffering from polyneuritis 9·31 % nitrogen and 1·53 % phosphorus. These figures correspond to a loss of a larger proportion of the phosphorus than of the nitrogen of the brain, which might be expected if the disappearing substance were of a lipoid nature. This marked difference leads to the conclusion therefore, that polyneuritis is caused in the first place by a lack in the food of this basic substance which is necessary for the normal metabolism of the nervous tissues. As the result the lipoids of the medullary sheath within and without the central nervous system are more or less disintegrated. On the other hand the analysis of the brain of cured pigeons (Table III) killed 24 hours after the cure shows that the resumption of function in the axis cylinder is not immediately followed by reparation in the medullary sheath.

Experimental. After the pigeons were killed the brain was taken out directly and dried in a vacuum desiccator. The dried organ was finally powdered and dried at room temperature in vacuo to constant weight, and the nitrogen and phosphorus determined by the usual methods. The cured pigeons (Table III) were cured by different fractions of the milk- and yeast-lipoid and fractions from lime-juice[1] and killed 24 hours after the cure. Underfed pigeons were used as controls (Table IV) and were fed on 5 grm. maize daily until they lost between 17 and 27 % of their body weight. As shown, no appreciable loss of nitrogen and phosphorus in these brains was observed.

Summary.

In polyneuritis of birds produced by a diet of polished rice, the analysis of the brains shows a sensible diminution from the normal of nitrogen and phosphorus content, in a proportion which suggests a breakdown of the lipoids of the brain.

[1] The preparation of the extracts employed in Table III will be described in a later communication.

REFERENCES.

(1) Casimir Funk. This Journal, XLIII. p. 395. 1911.
(2) Casimir Funk. Trans. of the Soc. of Trop. Med. v. p. 86. 1911.
(3) MacLean. Ztschr. f. physiol. Chem. LIX. p. 223. 1909; Biochem. Journ. IV. p. 240. 1909.
(4) Schaumann. Arch. f. Schiffs- und Tropenhygiene Beiheft. 8. 1910.
(5) Schaumann. Trans. of the Soc. of Trop. Med. v. p. 59. 1911.

TABLE I.

Normal Pigeons.

No.	Weight	Brain wet	Brain dry	N. in %	P. in %	N. in grm.	P. in grm.
I	264	1·85	·4024	9·53	1·85	·0383	·0074
X	314	1·90	·3948	9·73	1·79	·0384	·0071
XI	335	1·95	·4082	9·74	1·63	·0397	·0066
XII	312	1·75	·3708	9·64	2·07	·0357	·0077
XIII	354	1·95	·4040	9·70	1·76	·0392	·0071
XXVI	457	1·90	·4035	10·32	1·91	·0416	·0077
XXIX	310	1·70	·3558	9·98	1·81	·0355	·0064
XXX	215	1·45	·3394	9·57	1·92	·0325	·0065
Average	320	1·80	·3848	*9·77*	*1·84*	·0376	·0070

TABLE II.

Pigeons suffering from Polyneuritis.

No.	Initial weight	Final weight	Brain wet	Brain dry	N. in %	P. in %	N. in grm.	P. in grm.
68	250	210	1·90	·3718	8·94	1·50	·0332	·0056
71	245	197	1·80	·3556	9·60	1·45	·0341	·0051
72	257	194	1·15	·2060	9·82	1·38	·0216	·0028
79	293	217	1·95	·3716	9·05	1·50	·0357	·0055
80	235	164	1·80	·3480	9·30	1·54	·0324	·0053
83	277	217	1·25	·2458	9·53	1·46	·0234	·0036
84	302	237	1·80	·3624	9·32	1·41	·0338	·0051
87	404	310	1·90	·4318	9·12	1·56	·0394	·0067
88	327	264	1·85	·4106	9·37	1·76	·0385	·0072
89	349	265	2·00	·4326	9·34	1·73	·0404	·0075
90	297	220	1·75	·3720	9·35	1·51	·0348	·0056
91	393	297	1·92	·4148	9·01	1·66	·0373	·0069
Average	300	232	1·75	·3602	*9·31*	*1·53*	·0337	·0055

TABLE III.

Pigeons cured of Polyneuritis by different means.

No.	Initial weight	Final weight	Brain wet	Brain dry	N. in %	P. in %	N. in grm.	P. in grm.	
85	241	173	1·45	·3016	9·22	1·45	·0278	·0044	Phosphotungstate precipitate decomposed from hydrolyzed yeast-lipoid.
86	273	229	2·00	·4260	9·19	1·83	·0391	·0078	,, ,, ,, ,,
92	473	404	2·10	·4592	9·45	1·47	·0434	·0067	$AgNO_3$ salt from the milk lipoid.
95	375	315	2·14	·4548	9·28	1·11	·0422	·0050	Alcoholic extract from yeast.
96	233	186	1·70	·3570	9·56	1·72	·0341	·0061	$AgNO_3$ salt from yeast lipoid.
97	371	315	1·85	·4078	9·40	1·71	·0383	·0070	,, ,, ,,
98	235	195	1·55	·3338	9·54	1·71	·0325	·0057	$AgNO_3$ salt from lime-juice.
Average	314	259	1·82	·3914	*9·37*	*1·57*	·0367	·0061	

TABLE IV

Pigeons fed on insufficient food.

No.	Initial weight	Final weight	Loss of body weight	Brain wet	Brain dry	N. in %	P. in %	N. in grm.	P. in grm.	Food
XVII	327	240	26 %	2·00	·4317	9·56	1·69	·0413	·0073	5 gms. maize, daily.
XVIII	364	264	27 %	1·85	·4332	9·39	2·06	·0407	·0089	,, ,,
XIX	367	305	17 %	2·10	·4342	9·92	1·82	·0431	·0079	,, ,,
Average	352	269	23 %	1·98	·4330	*9·62*	*1·85*	·0417	·0080	

THE ETIOLOGY OF THE DEFICIENCY DISEASES.

BERI-BERI, POLYNEURITIS IN BIRDS, EPIDEMIC DROPSY, SCURVY, EXPERIMENTAL SCURVY IN ANIMALS, INFANTILE SCURVY, SHIP BERI-BERI, PELLAGRA.

BY

CASIMIR FUNK, PH.D.

THE diseases mentioned above present certain general characters which justify their inclusion in one group, called deficiency diseases. They were considered for years either as intoxications by food or as infectious diseases, and twenty years of experimental work were necessary to show that diseases occur which are caused by a deficiency of some essential substances in the food. Although this view is not yet generally accepted, there is now sufficient evidence to convince everybody of its truth, if the trouble be taken to follow step by step the development of our knowledge on this subject. This article is written with the intention of giving a summary of the modern investigations, and by means of a careful selection of references to facilitate the research for anybody who wishes to read the original literature. This careful selection was absolutely necessary, for there is perhaps no other subject in medicine where so many contradictory and inexact statements were made, which instead of advancing the research retarded it by leading investigators in a wrong direction.

The deficiency diseases break out in countries where a certain unvarying diet is partaken of for long periods. When this food happens to be deficient in a substance which is necessary for the metabolism, we have the real conditions for the outbreak of this type of disease. From this point of view it is surprising to see peasants in Russia and in other countries, who live on potatoes, cabbage, and a little bacon nearly exempt from these diseases; it will be seen later, however, that this one-sided food contains apparently all the protective bodies which are necessary.

All these diseases present some general characters, which may be sketched here. The most prominent symptoms are a general cachexia with an enormous loss of weight; marked nervous symptoms are often present, which are due probably to the

degeneration of the peripheral nervous system. It is now known that all these diseases, with the exception of pellagra, can be prevented and cured by the addition of certain preventive substances; the deficient substances, which are of the nature of organic bases, we will call "vitamines"; and we will speak of a beri-beri or scurvy vitamine, which means a substance preventing the special disease. As regards the classification two different groups present themselves : the beri-beri group and the scurvy group. The investigations made on pellagra, however, have not yet resulted in a sufficient elucidation of its etiology to establish it as a deficiency disease and it is included here provisionally owing to its similarity in some respects to the other diseases mentioned.

The Beri-beri Group.

To this group, which is characterized by more or less distinct neuritis symptoms, belong beri-beri, polyneuritis in birds and epidemic dropsy. Beri-beri occurs in countries such as Japan, Malay States, Philippine Islands, Indo-China, &c., where rice is used as a staple diet. This diet, however, must be eaten for long periods (six to seven months) to produce the disease. The symptoms which are described in most of the textbooks of tropical diseases (1) present several distinct types, which can be shortly summarized as follows. In most cases the patients lose enormously in weight, and very often suffer from œdema, contractions, paralysis and anæsthesia in the limbs. Pathological changes have been found such as degeneration in the nerves and heart. The disease, in most of the acute cases, terminates fatally. It has often been stated that sometimes the patients recover without any treatment. It is most probable in this case that the recovery is associated with some very important changes in diet. The investigators who have suggested a causal connection between beri-beri and an exclusive diet of rice are Wernich [2] and van Leent [3]. Acting on these ideas Takaki [4] was able by a change of diet (addition of meat) practically to eradicate this disease from the Japanese navy. Eykman [5] and his collaborator Vordermann [6] (especially the latter) came to the conclusion, based on an investigation in 101 jails in Java made in 1895-1896, that the disease has a distinct relationship to the continuous consumption of decorticated (polished) rice. This statement was confirmed on a very large scale by Braddon [7], who found that natives (such as Indians, Tamiels)

who use parboiled (cured) rice, which has been previously steamed to remove the pericarp, were not in danger of contracting the disease. Finally Fraser and Stanton [8] confirmed the results of the previous workers especially as to the harmlessness of parboiled rice.

The next step in Eykman's [9] investigation, established the important fact that birds (fowls, pigeons, ducks) when fed on polished rice developed a disease which he called polyneuritis gallinarum. The birds lose considerably in weight, and after three to four weeks, contract a disease which is very similar to human beri-beri. He also found that it was not possible to induce the disease with rice containing the pericarp or that part of the pericarp which is called the silver-skin by the Dutch authors. This most valuable observation facilitated enormously the further experimental research, and really started the experimental investigation. Eykman, in endeavouring to explain the origin of the disease, arrived at the conclusion that food rich in starch, like rice or starch itself, produced a substance in the intestine (pp. 526 and 527), which acted as a poison on the nerve-cells. He was, further, able to show that the silver-skin is richer in nitrogen than any other part of rice, and contains an antidote for the starch poison. He also stated [10] that the aqueous extract from rice-polishings (silver-skin) cures after the elimination of phytin, and that the protective substance dialyses and is not precipitated on alcohol. Gryns [11] confirmed Eykman's results, and to him belongs the credit of being the first worker to adopt the deficiency theory for the explanation of the etiology of this disease. He says clearly that the disease breaks out when a substance necessary for the metabolism of the peripheral nervous system is lacking in the food. He has discovered similar protective substances, in katjang-idjoe beans (*Phaseolus radiatus*) and meat, and showed that these foodstuffs lose their protective power when heated to 120° C. These heating experiments which were of great importance for the further knowledge of deficiency diseases were confirmed by Eykman [10]. Bréaudat [12] used rice-polishings as a remedy in cases of human beri-beri and obtained very good results. Fraser and Stanton [13] tried to characterize more closely the protective substance, and have found that it is soluble in strong alcohol and that this solution is effective after the elimination of alcohol-soluble proteins. From an analysis of different rices which induced beri-beri, they came to the

conclusion that a valuable practical indication as to whether the rice is harmful or harmless is its content of phosphorus. They found that the less the phosphorus content, the more liable it is to cause disease. On this basis Schaumann [14] constructed a theory, the phosphorus-deficiency theory, which he extended to other deficiency diseases such as scurvy and ship beri-beri, and which suggests that these diseases are due to a deficiency of certain organic phosphorus compounds in the food. With slight modifications he still holds this view.

During the last two years a quick succession of papers appeared which deal with the isolation of the protective substance from the different foodstuffs. Hulshoff Pol [15] found that an aqueous extract of Katjang-idjoe beans still retained its curative properties after the precipitate, obtained with lead acetate, was removed. From this filtrate he obtained a crystalline substance, called by him X-acid; he did not, however, show that this substance was actually capable of curing the disease. Schaumann [16], who added yeast to the series of curative substances, tried the different known constituents of yeast, such as yeast nucleic acid and lecithin, without, however, obtaining satisfactory results. Lately it was shown by Eykman [17] that the protective substance in yeast is soluble in 88 per cent. alcohol. Before this paper appeared the writer[1] tried to extract yeast completely with alcohol. The experiments failed, however, only a small part of this substance being extracted by this process, and even after repeated boiling for several hours with alcohol the extracted yeast still retained its curative properties. This shows that the bulk of the substance is present in the yeast in a combined state. Teruuchi [18] extracted rice-polishings with weak hydrochloric acid, and, after neutralizing to remove phytin, the solution was evaporated and extracted with alcohol. The solution obtained in this manner was active, although it contained only a small part of the total phosphorus content. Similar results were obtained by Chamberlain and Vedder [19], and Shiga and Kusama [20]. The latter have found that the protective substance of the rice-polishings is destroyed at 130° C. in 0·5 per cent. hydrochloric acid, or in 1 per cent. sodium carbonate solution, but not at 100° C. The rice-polishings being particularly rich in phytin, Aron and Hocson [21] performed some experiments

[1] Notes not yet published.

with this substance, and claim to have obtained satisfactory results. Their statements, however, are in discordance with those of Eykman (*loc. cit.* [11]), and could not be confirmed by Schaumann (*loc. cit.* [16]), and Cooper and Funk [22]. Kilbourne [23] suggested that the deficient substances might be salts, especially potassium salts, without, however, giving any experimental support to his theory.

In spite of all this experimental evidence which classifies beriberi beyond any doubt as a deficiency disease, Kohlbrugge [24] lately isolated a micro-organism from rice, which he calls *B. oryzæ*. The culture, when injected into fowls, was capable of producing polyneuritis in five days. The weak point of his theory is his explanation of the efficiency of rice polishings in preventing the disease. He states that the *B. oryzæ* produces a special kind of fermentation, which is stopped by the acid formed from rice-polishings. The epidemical occurrence of beri-beri suggested to many authors that infection is the real cause. It has been stated that by transferring patients from one pavilion to another they suddenly recovered. A careful inquiry showed, however, that this transfer was associated either with a change in diet or with a change in the cooking apparatus.

Summarizing our knowledge till 1911 of the chemical nature of the protective substance from the rice-polishings, we find the following well-established facts :—

(1) The substance is soluble in water, in alcohol, and in acidulated alcohol.

(2) The substance is dialysable.

(3) It is destroyed by heating to 130° C.

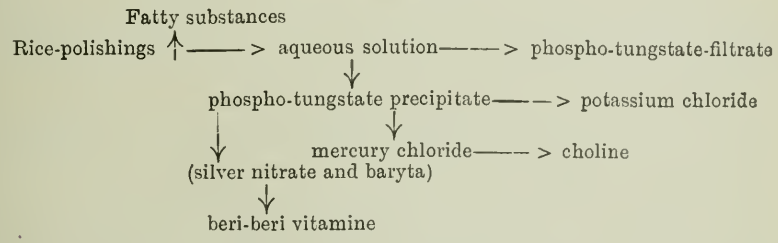
(4) Is neither a salt nor a protein.

We see, therefore, that our knowledge of the chemical nature of the substance was very small. This was the state of the subject when I began my experiments in the middle of 1911, first in collaboration with Cooper (*loc. cit.* [22]), and afterwards by myself. The first problem which had to be settled was to ascertain to what class of chemical bodies this peculiar substance belonged, and whether it was a complicated compound or one of a comparatively simple structure. Is it easily destroyed by chemical manipulations? This last condition was a *sine qua non* for further chemical investigation.

By a series of experiments with yeast it was possible to show that dried, pressed yeast hydrolyzed for twenty-four hours with

20 per cent. sulphuric acid still retained its curative properties. Based on this remarkable stability in acid solution I came to the conclusion that the curative substance might be one of the nitrogenous substances, which are precipitated by phospho-tungstic acid, and that it is of a simple chemical nature. A systematic investigation of rice-polishings was then started on these lines (*loc. cit.* [25]), and very satisfactory results were obtained. The different fractions separated during this investigation were tested on pigeons which were suffering from polyneuritis induced by a diet of polished rice. These pigeons presented very marked symptoms consisting of cachexia, weakness of the wings and legs, and a peculiar spastic paralysis of the neck, which in many cases caused the head to be bent back towards the tail of the animal. Left to themselves pigeons in this condition died as a rule in less than twelve hours. The various fractions obtained during the numerous manipulations were introduced by means of a syringe into the crop. The method used is shortly summarized as follows: The polishings were extracted on a shaking machine for several hours, with absolute alcohol partially saturated with gaseous hydrochloric acid. After removal of the alcohol *in vacuo* a fatty residue remained, which was melted and extracted with water on the water-bath; the aqueous fraction was then separated off in a separating funnel in the hot chamber at 37° C. This aqueous solution, which was capable of curing pigeons, was precipitated with phospho-tungstic acid. The separated phospho-tungstate precipitate was decomposed with baryta, and the solution, after removing the excess of baryta, tested on pigeons. Here the first experimental difficulty occurred. It was at first impossible to ascertain the quantity necessary for curing pigeons. In my first experiments too large a dose was administered, and the pigeons died in a few minutes with marked toxic symptoms. We will see later that this was due to the presence of choline. This difficulty can be avoided by calculating the dose on the quantity of polishings used. We know that 20 grm. of rice-polishings or the aqueous extract of this contains sufficient substance to cure pigeons. All the later fractions were administered in doses corresponding to the amount of original polishings necessary to cure, a small allowance being made for unavoidable losses due to the necessary manipulation. The above-mentioned solution was totally free from phosphorus, proteins and carbohydrates. This fact simplified the chemical task considerably,

and indicated at the same time that the essential substance was of basic nature. This fact spoke against Schaumann's phosphorus deficiency theory, for it was clearly established for the first time that cures could be effected by solutions which contained no phosphorus. Since the filtrate from the phospho-tungstic acid precipitate was shown to be entirely devoid of action, the whole of the curative substance must have been in the precipitate. This precipitate appeared to be very simple from the chemical standpoint, as only potassium and choline could be detected. By precipitation with gold- and platinic-chloride no other basic substances could be detected, and experiments showed that choline was devoid of action. After removing all the choline by means of platinic chloride in alcoholic solution, the filtrate still retained its curative properties. The presence of another basic substance was therefore inferred, and various reagents were used to precipitate the latter. After many trials it was found that the substance could be precipitated by means of silver nitrate and baryta. The silver salt obtained proved to be curative, and by decomposing a crystalline substance was obtained, which melted at 233° C. The analysis of this compound led to the formula $C_{17}H_{20}N_2O_7$. The unsaturated nature of this compound would explain very well its physiological instability in heating experiments. So far the few experiments performed have shown that this substance seems to be the curative agent, and for purpose of simplicity I would propose to call it provisionally beri-beri vitamine. The method of fractionation is indicated in the following table :—



The yield of vitamine was extremely small. From 1 kg. polishings only ½ grm. of crystalline substance was obtained. This is the chief experimental difficulty in the research. It is hoped, however, that an extraction on a very large scale will give the opportunity of investigating this most interesting substance more closely.

The dose of the crystalline vitamine necessary for curing pigeons was very small. Such a minute dose as 40 mg. of the substance was not only sufficient to cure a pigeon in a very short time—often in three hours—but also maintained the cured animal in health for periods varying from seven to twelve days, when polished rice was used as food. After this time symptoms of the disease again manifested themselves.

Schaumann [26] expressed the idea that the curative agent acts only as an activator, and that besides this a substance of a phosphatide nature is necessary. He thinks that these minute quantities cannot be considered as food, but can only act as hormones. This can be settled by direct investigation. It is necessary to perform experiments on pigeons fed on polished rice with the addition of vitamine. These experiments will be performed when a sufficient quantity of pure substance is obtained. With the discovery of this substance it was possible to try to explain the action of these protective substances. It is surprising how rapidly the nervous symptoms disappear after the administration of vitamine. This naturally suggested that this substance is wanted for the metabolism of the nervous tissues and has some connection with cell lipoids. It was therefore thought useful [27] to compare the contents of nitrogen and phosphorus in the brains of pigeons fed on polished rice with that of normal pigeons. This investigation [28] showed that the brain of the polyneuritis pigeons was sensibly poorer in nitrogen and phosphorus compared with the normal ones, a fact which suggests a degeneration of the brain lipoids. Palladino [29] has shown recently that the same happens in the brain of starved dogs. It is interesting to mention here that Chamberlain, Bloombergh, and Kilbourne [30] have sometimes observed marked neuritic symptoms in starvation in birds. It is not surprising that in starvation the same deficiency occurs, but it is probable that very often the general weakness develops before the neuritic symptoms have time to appear. As a matter of fact, by using the voluntary feeding method instead of forced feeding I often noticed that the pigeons refuse to eat anything in the last period and soon show characteristic neuritic symptoms. Coming back to the chemistry of brain, I was surprised at the small normal nitrogen content of pigeons' brains. The relationship between the nitrogen content of the administered dose and the total nitrogen of the brain, or even of the total lipoid

fraction of the body, is so small that we might eventually consider the vitamine as a specific food supply for this special kind of tissue.

In search of foodstuffs which would give a better yield of vitamine other substances were investigated, and bodies capable of curing beri-beri could be detected in milk, yeast, ox-brain and lime-juice (see under Scurvy). The method[1] used here was analogous to that used in the case of rice-polishings. The cure with fractions obtained from ox-brain was so unexpectedly quick that it seems possible that the substance contained in brain is ready for use. It is obvious from the statements of previous workers that similar substances are present in vegetables, cereals and meat. It is interesting to note that the vitamine can be extracted from all these substances by means of alcohol. However, in the cases investigated by me the vitamine could be extracted from the alcoholic residue with water.

Another problem as to the form in which the vitamine is present in the food presents itself here. Is it in the form of a lipoid and split off by simple contact with alcohol, or is it only absorbed to the lipoid fraction? This interesting question requires further consideration. Even if the vitamine is combined in a lipoid molecule, it is not necessary to accept Schaumann's view. As long as the organism is in a healthy state it does not matter, according to the usually accepted conception of the breaking down of food into simpler products in the intestine, whether it gets a supply of a lipoid or of vitamine itself.[2] As the phosphoric acid is present in polished rice in large quantities and fatty acids can be eventually built from carbohydrates, the organism could easily synthetize its own lipoid. If the lack of vitamine occurs and the body is not able to synthetize this, and must get it ready from plants, then deficient metabolism ensues and death results. It was stated by Schaumann (*loc. cit.* [26]) that yeast cures better than anything else tried up to now. I suppose that in a state of general cachexia the synthetical power declines. Here the daily supply of vitamine to the deficient food will elucidate this problem entirely.

The author's view on the cause of beri-beri may be summarized

[1] Not yet published.

[2] Compare here the paper by Fingerling [31], who finds that the organism is even able to use inorganic phosphates for the synthesis of lipoids.

as follows (the physiological importance of the vitamine will be discussed in the last chapter). Judging from the manifest nervous symptoms, the fatty degeneration in the nerve-cells, and the above-mentioned chemical changes, I am inclined to think that vitamine is necessary for the metabolism of the nervous tissue. The lack of vitamine in the food forces the animal to get this substance from its own tissues. The result is an enormous loss of weight. After this available stock begins to be scarce there is a consequent breaking down of the nervous tissue, with the result that nervous symptoms, such as are observed in beri-beri, manifest themselves.

In the beri-beri group of deficiency diseases we may include epidemic dropsy, which is called by the French authors the wet form of beri-beri. This disease occurs in India, and Grieg's [32] investigation suggests that it is due to the same cause as beri-beri —namely, to an exclusive diet of polished rice.

Scurvy Group.

(*Scurvy, Experimental Scurvy in Animals, Ship Beri-beri, Infantile Scurvy.*)

Scurvy.—As characteristic clinical symptoms of scurvy we find very often anæmia and general cachexia, a marked tendency for local hæmorrhages and hæmorrhagic inflammation, especially in the gums. In adults often hæmorrhages in the periosteum and in the frontal endings of the ribs, with changes in the marrow of the bones, followed by a dislocation of the ribs from the cartilage. Lately cases with neuritis and anæsthesia of the limbs were observed by Holst [33].

It is now generally accepted that scurvy is caused by the food used. A diet consisting chiefly of starch, bread and tinned preserved food is especially liable to produce the disease. The remedy for this disease, as found by sailors in an empirical way, consists in good dietetical conditions, lime-juice, fresh fruits and vegetables, especially onions. Scurvy patients recover very quickly when these articles are available.

Just as in the investigation of beri-beri, the discovery of experimental scurvy by Holst [34] and his collaborator Frölich was of great importance. They found that many animals (guinea-pigs, rabbits, pigs, and dogs) kept on scurvy-producing diet, or on food known by experience to be capable of producing scurvy, or on

food autoclaved at high temperature, develop a disease which is closely analogous to human scurvy. These authors have observed that guinea-pigs fed on water and rye, or wheat bread (or oats, rye, wheat, barley and rice-flour), or on barley (peeled grain), from oats, barley or rice, die after several weeks of this diet. If death occurs after three weeks the following changes were found constantly: The side teeth were loose, with hæmorrhages in the gums; sometimes only a blue hyperæmia was observed, and in few cases ulceration; hæmorrhages were found constantly in the periosteum of the ribs.

For the investigation of scurvy Holst particularly recommends guinea-pigs, fed on oats and water. For such experiments only grown-up animals weighing over 350 grm. should be used. The animals should be weighed every few days, as they lose considerably in weight (about 40 per cent.). Similar experiments were made by Schaumann (*loc. cit.* [16]), who experimented especially on dogs and monkeys, by feeding them on food taken from sailing ships, where ship beri-beri broke out, or with food extracted previously with sodium carbonate. We will see further on the great similarity of the latter with scurvy.

The great experimental difficulty here was, that the scurvy protecting substance, or as we call it scurvy vitamine, is much less stable than the corresponding beri-beri vitamine (Holst) [35]. As protective food in the experiments of Holst and Frölich fresh potatoes, apples, lime-juice, carrots, cabbage and leaves of lions-tooth (*Leontodon taraxacum*) were used. The addition of this food even protected guinea-pigs which had been fed already for three weeks on oats. However, only fresh food possesses the protective power; dried material is completely valueless. This fact explains, largely, why food kept for a long time on ships produces scurvy. A guinea-pig fed on fresh potatoes dies after several months without scurvy, whereas one fed on boiled and dried potatoes dies after a few weeks with characteristic scurvy symptoms. No difference was observed if the drying process was performed in vacuo or in air. The same thing applies to carrots. Cabbage heated to 110° C. loses completely its protective power, and the juice obtained by pressing the cabbage was inactivated even at 60° or 70° C. The same occurs if the juice is kept for a couple of months, even with the addition of antiseptics.

Different in this respect is lime-juice. Although comparatively

poorer in vitamine, the latter seems to be much more stable; it is not destroyed even after boiling for one hour. A set of interesting experiments were described by Fürst [36] (from Holst's laboratory). He tried the effect of food, yielding an alkaline ash (it was often stated that scurvy is a result of a special kind of acidosis). This food failed to prevent scurvy, so did the addition of yeast. The last fact is remarkable, as it is known that yeast prevents beri-beri very well. This fact will be discussed in the following chapter. Fürst further describes some experiments with seeds. Grains which are known to prevent beri-beri such as oats, develop an antiscorbutic agent after they have been germinated. They lose this power when they are dried again, and in the presence of moisture develop the scurvy vitamine. In the process of germination seeds are known to develop all kinds of enzymes in order to utilize their food reserves. This fact suggested to Fürst that this substance might be produced by the action of enzymes, and he suggested that on board ship a store of dried grain should be kept, which after germination should be added to the diet on long voyages, when no fresh provisions could be provided. It is surprising that a chemical change, which can be performed by the enzymes of the seed, cannot be reproduced by the animal body, which seems not to be adapted for this kind of reaction. From this short *résumé* we see that the material which promises the best results in the way of chemical investigation with regard to vitamines is lime-juice. The investigation of vegetables, owing to the feeble stability of their vitamine, do not promise good results. Some other points also suggested to me to work with lime-juice. It possesses the advantages of being easily obtained and contains substances in a soluble state, which makes the experimental work comparatively easy. Although the original commercial lime-juice does not cure polyneuritis pigeons (as was also stated by Chamberlain and Vedder, *loc. cit.* [19]) I was encouraged to persevere with it by its fairly high nitrogen content, which amounts to 0·35 per cent. This suggested to me that a fractionation, analogous to that done with rice-polishings, might give interesting results. The investigation is not finished yet and will be repeated again on a large scale. The first fractionation was done on 50 litres and the results were very promising. Here, as in rice-polishings, the same difficulty occurs—the minute quantities of the vitamine present. The lime-juice was cleared by precipitation with lead acetate, and the filtrate, evaporated *in vacuo*, was precipitated

with phospho-tungstic acid. Although the original lime-juice was unable to cure polyneuritis pigeons, this was done by the decomposed phosphotungstate precipitate. However, the effect in some cases was not as good as with the similar fractions from rice-polishings; apparently here the question of dose enters. By means of silver nitrate and baryta two fractions could be obtained from this precipitate. The experimental work, which has to be repeated again, gave the following results : The first fraction, which could not be obtained crystalline, was effective in curing polyneuritis pigeons. I was surprised to see that the same fraction, after my return from holidays, lost its curative properties after six weeks. The second fraction yielded 0·4 grm. of a crystalline substance, which, administered to polyneuritis pigeons, kept them alive for seventy-two hours without, however, improving their condition. This minute quantity of substance was used up in two experiments on guinea-pigs fed on oats; into each guinea-pig a solution of this substance was injected subcutaneously every second day in the period, when they began to lose weight. No definite results have been obtained up till now. From these experiments I am inclined to think that scurvy vitamine is different from the beri-beri vitamine, although chemically belonging to the same class of substances.

Ship Beri-beri. — This disease, which somewhat resembles scurvy, was carefully investigated by Nocht [37], and takes an intermediate place between scurvy and beri-beri. As described by Nocht, this disease breaks out on board sailing ships on long voyages when fresh provisions begin to be scarce. Characteristic symptoms are anæsthesia, short breathing, and in fact death from heart weakness. After a supply of fresh food the patient recovers very quickly, in contrast to tropical beri-beri. Neuritis symptoms were described only in few cases. Holst and Fröhlich [34] consider this disease to be very closely related to scurvy, and it would appear that all that we know concerning the etiology of scurvy applies equally well to ship beri-beri.

Infantile Scurvy.—Infantile scurvy breaks out in children fed on sterilized milk, with or without the addition of different flour preparations. For the first exact description of this disease we are indebted to Sir Thomas Barlow [38]. The pathological changes were especially investigated by Fränkel [39], who describes changes in the bone-marrow, which loses and becomes poor in cells, as a result of the defective new formation of the bone;

further, he describes a separation of the epiphysis and alterations in the intermediate cartilage and the cartilages of the ribs. In children in which the teeth were present hæmorrhages and affections of the gums were observed. This description indicates the similarity of these symptoms with those present in human scurvy cases, and especially with those described in the experimental scurvy of the guinea-pig. This close similarity of symptoms led Sir Thomas Barlow, Axel Holst [35] and Looser [40] to conclude that infantile scurvy and scurvy are identical. Recently Vortisch van Vloten [41] published a paper, in which he says that both diseases are identical and caused by a deficiency of nutritive salts, especially potassium and iron.

Clinical interest in this disease was considerably increased by the interesting communication of Neumann [42], who first showed that infantile scurvy had some connection with boiled milk. He found in a large number of cases that children fed on milk, previously heated in Soxhlet apparatus for ten to fifteen minutes, got infantile scurvy. His explanation was that during the boiling process toxic substances were formed in the milk. Heubner [43] was able to confirm these results. Corroboration of these statements has been furnished by data collected by A. Meyer [44]. Cases of infantile scurvy due to boiled milk were observed recently in this country by Brachi and Carr [45]. The patients can be cured either by an addition to the food of unboiled milk or of lime or fruit juices. As Holst and Fröhlich have found, these contain the antiscorbutic substance.

Is there any evidence that milk contains a substance which is destroyed by boiling? This question must be answered in the affirmative. Bordas and Raczkowski [46] have found that lecithin is partially destroyed during the boiling of milk, even at 60° C. a fair amount disappears, at 95° C. 28 per cent., and after heating for half an hour at 105° to 110° C. as much as 30 per cent. is destroyed.

Bartenstein [47] observed that guinea-pigs fed on boiled milk contract a disease which he considers to be identical with infantile scurvy. Fröhlich [48], who repeated Bartenstein's experiments, does not quite agree with the latter; he states that guinea-pigs fed even on raw milk show, with the exception of slight tendency to a porosity of bones, a normal aspect, whereas those fed on milk previously heated for ten minutes at 100° C., thirty minutes at 100° C., and one hour at 112° C., show a high fragility of the bones,

which is a characteristic of scurvy, but without hæmorrhages and changes in the ossifications.

Besides that, Fröhlich (*loc. cit.*) [48] performed a series of experiments on the protective power of milk on guinea-pigs fed on oats. He found that pasteurized milk (heated at 70° C.) prevents scurvy, and milk heated for ten minutes at 98° C. loses its protective power completely.

Stepp [49] found that mice fed on food which was extracted with ether died, while the addition of the extract kept the animals in good health for a long time. Milk was shown to contain the necessary substance in small quantities and was inactivated by boiling. The animals could be kept also by an addition of ox-brain lipoid.

Some preliminary experiments done by the author[1] demonstrated the presence of the beri-beri vitamine in milk, its commercial preparation known as "Trumilk" was used for this purpose and extracted with alcohol and ether. After hydrolysis of the lipoid the vitamine was separated by means of phosphotungstic acid and silver nitrate and baryta. A small quantity of a nitrate of a base was thus obtained which was capable of curing polyneuritis pigeons. The scurvy vitamine could not be detected. The probable cause of this was that during the preparation of "Trumilk" the milk was exposed to a high temperature whilst being concentrated *in vacuo*.

This vast amount of evidence suggests that the real physiological difference between the raw and boiled milk is not in the destruction of enzymes, antibodies and changes in proteins, but in the content of vitamine. The scurvy vitamine in the milk seems to be a fairly stable one. It is not destroyed like that in cabbage at 60° to 70° C., but resembles more that of lime-juice. The scurvy vitamine, as we know from other foodstuffs, is certainly destroyed at 120° C., but even at lower temperature the quantity of vitamine destroyed depends on two factors—the temperature used and the time of heating. Even in this case probably only a fraction of the vitamine is destroyed. The reaction of the milk, the natural content of the vitamines in cows' milk, which of course depends entirely on the content of vitamines in the cows' food, are further factors of which we do not know the importance.

[1] Not yet published.

Recently Miss Lane-Claypone, in a report to the Local Government Board,[1] came to the conclusion, on the basis of the clinical evidence, that there is no real evidence to support the view that infantile scurvy is due to boiled milk. The reasons mentioned above—the influence of the time of heating and the vitamine content of the original milk—render the clinical observations of relative value only. In addition to this it is often impossible to perform exact experiments in the hospital, and therefore besides clinical evidence the results of experimental research on animals ought to be collected.

At present our knowledge does not go beyond the fact that milk is a food which provides all the constituents necessary for the growing organism, and must, of course, contain all kinds of vitamines. All investigations bear out this view. On the other hand, we know that these substances are very unstable and destroyed by heat. As a result of this I would suggest that it is safer not to heat the milk more than is strictly necessary.

The Relationship between Beri-beri and Scurvy.

Between these two diseases a close analogy undoubtedly exists. In the siege of Paris, cases of scurvy were described by Delpech [59], which arose from an exclusive diet of white rice. Similar observations were recorded by Bucquoy [50]. Experiments on animals show that rice produces in some animals (pigeons, fowls, ducks, &c.) polyneuritis, in others, such as guinea-pigs and dogs, scurvy. For instance, pigs under these conditions show symptoms of neuritis and scurvy (Holst, *loc. cit.* [33]). On the other hand, Holst describes cases of scurvy with marked neuritis. In spite of all this evidence both diseases have to be considered as entirely different. The common cause in both is a deficiency in diet, but a deficiency of two different substances. Many facts are known which speak in favour of this idea. We saw that the beri-beri vitamine is a much more stable substance than the scurvy vitamine. Different foodstuffs, which are known to prevent beri-beri and polyneuritis, like yeast, oats and barley (unhusked), are unable to prevent scurvy. On the other hand, grains develop the scurvy vitamine during germination. This fact suggests that the scurvy vitamine can be formed by enzyme action from the beri-beri vitamine, which is undoubtedly present in these seeds. This would

[1] Reports to the Local Government Board, New Series, No. 63, 1912.

suggest a certain connection between the two substances. In favour of this view many facts are on record, but the problem can be easily solved by experiment. It is necessary to investigate whether this process goes hand in hand with the diminution of the beri-beri vitamine in the seeds. I intend to perform such experiments shortly. The complete investigation of lime-juice, in which both substances occur, may throw some light on this interesting problem. The nearer the chemical constitution of both substances the closer will probably be their physiological connection.

From the experiments on germinated seed, performed by Fürst (*loc. cit.* [36]) we saw that the animal organism is not able to perform the slight change which is brought about by enzyme action in the grain. From this it is obvious that the anti-scurvy substance must be supplied as such by plants. The outbreak of scurvy as a result of a diet which is known to contain the beri-beri vitamine suggests that the organism is not able to do this kind of transformation. But the opposite reaction (the formation of beri-beri vitamine from the scurvy vitamine) is possible, and there are no facts known which disprove this suggestion. In this case we would obtain a cycle, which may be sketched here :—

Beri-beri vitamine———>plant enzymes———>scurvy vitamine.
Scurvy vitamine———>animal organism———>beri-beri vitamine.

By this means the scurvy vitamine would prevent both diseases, whereas the beri-beri vitamine only beri-beri. As a matter of fact, this suggestion explains very well indeed the occurrence of ship beri-beri. When both substances are deficient, then the disease which we know under the name of tropical beri-beri breaks out. On ships the food gets poorer in scurvy vitamine quicker than in beri-beri vitamine. The result of this is an outbreak of scurvy or ship beri-beri. This, however, is only an attempt to explain the close relationship between these two disease.

In conclusion, we might say that beri-beri and scurvy are both caused by a deficiency of diet. The deficient substances, though different, are closely related, and transformable to a certain extent into each other. The influence of this deficiency on the general metabolism will be dealt with in the last chapter.

Pellagra.

The investigation of this disease moves now essentially on bacteriological lines. From the history of beri-beri and scurvy

investigation we saw, however, that many years of careful study were necessary to establish the true etiology of this disease. The long struggle between the believers in the theories of infection and intoxication is worth mentioning, and still persists in spite of modern results. The idea that pellagra is due to some deficiency in the diet was expressed by several authors, but at present there is no positive evidence in favour of this theory, as against any other theory. A glance at all the existing theories suggests that an investigation of this disease on the lines mentioned above for beri-beri and scurvy might yield valuable results. Up to now the only evidence which speaks in favour of this view is its close analogy with beri-beri, and especially with scurvy. Research on this subject, which in the past has been very one-sided, is rendered more difficult by the impossibility of producing experimental pellagra in animals, and also by the lack of knowledge on the prevention of this disease by means of a change of diet.

Pellagra, which was known in Italy in the eighteenth century under the name of scurvy or Italian scurvy, occurs in Italy, Roumania, Austria, Spain, Portugal, Egypt, Algeria, United States, Mexico and Central America, and is strictly limited to districts where maize is used as the staple diet. This fact, along with the observation that the disease breaks out in countries where maize was recently imported, speaks clearly in favour of some relationship between pellagra and maize. This disease, which breaks out mostly in spring and autumn, shows nervous and psychic symptoms, and leads often to general cachexia, diarrhœa, and suicide. A very characteristic symptom is an erythema of the skin, which is caused by the sun and is localized in uncovered parts of the body, such as the hands, face, and bare feet.

In addition to such views as those expressed by Hodson [52], that pellagra is not a definite disease, there exist five distinct theories—namely, the intoxication, auto-intoxication, infection, photodynamic, and deficiency theories.

The *intoxication theory*, which expresses the view adopted in Italy for many years, was first suggested by Ceni [53] and Otto [54]. They observed that certain *Aspergillus* and *Penicillium* strains, which are constantly found in spoiled maize, secrete toxic substances, which, when isolated and administered to animals, provoked intoxication symptoms closely resembling those of pellagra. This theory was afterwards extended and energetically

advocated by Lombroso [55], followed by Gosio [56] and Gavina [57]. Lombroso especially performed a great number of experiments, and was able to isolate an alkaloid from these fungi which possessed a marked toxic effect resembling ergot poisoning. He considered the disease as a chronic toxæmia, due to toxic substances developed in maize by the action of micro-organisms, especially fungi. This theory is still accepted with slight modifications in Italy (Bertarelli) [58] and Antonini [59] and was recently expressed again by Camurri [60]. The latter accepts the view that besides an insufficiency of maize diet and lack of nutritive salts an intoxication takes place. He especially points out that ferments of maize, which he thinks capable of forming toxic substances, are able to act on maize, even after cooking. Acting on these views the Italian Government took measures to endeavour to eliminate spoiled maize from the market and to provide the population with drying apparatus: special hospitals for pellagra cases have also been established. All these measures seem to have very little effect, and the number of patients is still increasing.

The Auto-intoxication Theory.—Originated by v. Neusser [61] and de Giaxa [62], who stated that pellagra is due to toxic products formed in the intestine under the influence of certain bacteria, especially of *B. coli.*

The Infectious Theory.—An enormous amount of different micro-organisms have been credited with the power of causing pellagra, but only a few, which seem to have some claims based on a large amount of evidence, will be described here. Di Pietro [63] found a particular strain of *Penicillium glaucum,* to which he attributes the power of producing the disease. Ceni [64] isolated from the organs of pellagra patients *Aspergillus fluorescens* and *A. fumigatus.* Tizzoni [65] and Tizzoni and Panichi [66] describe a microbe, isolated from the organs and fæces of pellagrins, which they call *Streptobacillus pellagræ.* The same germ could be detected in spoiled maize. The culture of this microbe inoculated into guinea-pigs was, however, unable to produce the disease, unless the animals were put on a maize diet. Sambon [67], after a careful inquiry in Italy, came to the conclusion that pellagra is not necessarily associated with maize diet, and its topographical distribution (near to running streams) suggested to him that the disease may be caused by a protozoan transmitted by a biting fly from the genus *Simulium.* He was unable, however, to find the micro-organism,

Similar association with a Simulium fly was suggested in Georgia by Roberts [68].

Recently a very peculiar micro-organism was isolated from the cerebro-spinal fluid of the patients and described by the British Pellagra Commission [69], but has not been further investigated. Certain curative results obtained many years ago with arsenic, and recently with atoxyl, related by Babès and Vasiliu [70], and Babès, Vasiliu and Gheorghus [71] suggested a protozoological nature of the germ: good results with salvarsan were also recently related. The number of cured cases was relatively small and might be due to a change of diet.

Babès and Busila [72] were, however, unable to find a specific connection between the serum of the pellagrins and the micro-organisms isolated from the organs and fæces of the patients. The same negative results were obtained with extracts prepared from the micro-organisms of pellagrins or of maize. Tizzoni [73] recently, in a preliminary communication, describes a specific precipitation obtained by *Streptobacillus pellagræ* in the blood of pellagrins. Finally, Alessandrini [74], who observed a connection between pellagra and running water, in which larvæ of nematodes were found, accepts the latter as the cause of the disease.

Nevius-Hyde [75] criticizes all the evidence brought forward and states that the disease is produced by all kinds of spoiled flour. In a recent American report on pellagra by Clarke, Hamill, Pollock, Curtis and Dick [76], the authors come to the conclusion that there is actually no basis for the infection theory. They were unable to infect monkeys with the blood of pellagrins. Further criticisms of these theories will be found in the paper of Raubitschek

Photodynamic Theory.—Raubitschek [77], in his very interesting and critical paper, begins his research by investigating experimentally all the known theories, and comes to the conclusion that all these theories do not stand a careful experimental test. He found that polenta (a kind of maize-cake like that eaten in Italy) was almost sterile, and as a rule also the blood of pellagrins. The bacteriological examination, *post mortem*, gave no evidence whatever in favour of an infective etiology of pellagra, and the sero-diagnostic examination of the blood did not show the presence of antibodies against maize proteins or germs contained in maize. Opposed to the view of many Italian authors, Raubitschek found

that extract of spoiled maize inoculated into animals gave no trace of symptoms which could be considered analogous to those in pellagra.

Based on the experiments of Aschoff [78], who admits the existence of sensitizing substances against light in the lipoids, he makes the hypothesis that spoiled maize produces a toxic substance which is able to sensitize the skin for sun rays. Analogous experiments were already performed by Hausmann [79]. Raubitschek's experiments were carried out in the following manner: White mice fed on maize and exposed to daylight developed, after four weeks, a disease, with very marked loss of weight. After six to eight weeks the animals died, often with cramps and hyperæmia in the ears and mouth. White mice kept as controls in the dark or in light on a mixed diet, and dark-furred mice kept on maize in the light, did not show any trace of reaction. This disease is somewhat analogous to fagopyrismus, a disease which breaks out in white cattle fed on buckwheat. Raubitschek found that the sensitizing substance is present in the alcohol-soluble fraction of the maize. Maize extracted previously with alcohol was proved, then, to be harmless to white mice exposed to light, whereas the extract contained the toxic substance. The same results were obtained with spoiled maize (polenta) and rice. From the last fact Raubitschek concludes that beri-beri has a photodynamic origin. Similar observations were related independently by Horbaczewski [80] and Lode [81]. The results of these authors agree with those of Raubitschek. On the other hand, several objections were already made to this theory. Lavinder [82] was not able to confirm Raubitschek's results; Babès [83] does not believe that good maize is able to produce pellagra. Hausmann [84] points out that in time of intense sunlight the skin affection is less pronounced than in spring.

Raubitschek's experiments seem to apply only to albino mice, and do not show any analogies with the known experiments on the importance of lipoids or alcohol-soluble fraction for the life of animals (Stepp's experiments on white mice), in beri-beri and polyneuritis. Albino rats put on rice diet in daylight do not show any symptoms of disease for ten weeks.

The Deficiency Theory of Pellagra.—This short *résumé* shows very well that the present state of the pellagra investigation is the same as that of beri-beri ten years ago. One might be surprised

that the recent advances in our knowledge of beri-beri did not influence more the investigation of pellagra. It is beyond any doubt that pellagra has some close connection with maize diet. Even if cases of pellagra are known which have no relationship with maize, this would not speak against the deficiency theory, as long as the food used is also deficient. Unfortunately, I could not find many data about the diet in districts where pellagra occurs. Lombroso (*loc. cit.* [55]) and Camurri (*loc. cit.* [59]), however, describe such diet :—

Lombroso (p. 10).		Camurri.	
Maize	1,091 grm. a day.	Polenta	1,500 grm.
Beans	60 „ „	Milk	100 „
Rice and barley ...	67 „ „	Rice	100 „
Potatoes	67 „ „	Beans	100 „
Vegetables	250 „ „	Bacon	20 „
Pork meat and bacon	21 „ „	Vegetables	100 „
Olive oil	33 „ „	Potatoes	100 „
Fish	67 „ „	Cheese	50 „
Poultry	27 „ „	Olive oil	10 „

On the other hand, considerable changes in diet were observed during the different seasons. Here the following data would give an explanation of the occurrence of the disease in the spring and autumn. Lombroso (*loc. cit.*, [55] p. 11) reported about the change of diet in the Province of Ferrara :—

	Diet in eight winter months	Diet in four summer months
Polenta... ...	... 1,000 grm. ...	... 160 grm.
Milk	None ...	... None
Eggs	Nearly none ...	... Nearly none
Onions	One a day ...	... Two a day
Bread from maize ...	50 grm. ...	... 400 grm.
Home-made bread ...	50 „ ...	... 200 „
Meat	10 „ ...	... 60 „
Cheese	5 „ ...	... 20 „
Beans	150 „ ...	... 40 „
Fish	20 „ ...	... Very little

This diet is very one-sided, and consists chiefly of starch, which is known to produce beri-beri. We know, however, from our previous experience, that maize, beans, vegetables, milk and potatoes contain sufficient beri-beri and scurvy vitamine to prevent these diseases. We must, therefore, conclude that pellagra is due, probably, to a deficiency of a vitamine different from those men-

tioned above. Experiments carried out on animals with maize diet gave the following results :—

Bezzola [85] has shown that maize is not sufficient food to keep guinea-pigs in good health; they soon lose hair and get diarrhœa and die. No difference was observed whether good or spoiled maize was given. Lucksch [86] found that guinea-pigs fed on a good quality of maize lose their hair, show a hyperæmia of the intestinal mucosa, and an increase in size of the suprarenal glands. Fed on a mixture of maize, flour and green food they lose hair and get paralysis of the hind legs and a catarrh of the small intestine. The maize diet was, then, proved too insufficient also for rabbits and dogs. In springtime the results were more marked than in autumn. The blood of these animals was examined and found sterile. Holst [87] points out the very frequent occurrence of scorbutic symptoms in pellagra, especially the porosity of the bones, which is one of the characteristic symptoms of scurvy. He repeated the experiments of Lucksch on guinea-pigs, and stated that he could prevent the disease described by Lucksch, which he considers to be scurvy, by an addition of fresh cabbage. Loss of hair and other symptoms were absent in his cases, although the experiments were carried out in springtime.

Schüffner and Kuenen (*Arch. f. Schiffs- und Tropenhyg.*, vol. xvi, p. 277, 1912) have found an erythema of the skin in 23 per cent. of the beri-beri patients in Dutch India.

In order to ascertain definitely whether or not pellagra is a deficiency disease, exact investigation of food in pellagra districts are necessary. Further, it is important to know whether it is possible to improve the condition of the patients by any change of diet. Lombroso (*loc. cit.*[54]) in his book states that a considerable improvement and even cure could be obtained by a meat diet. These experiments, carried out on a large scale, with the elimination of possible errors, would probably give some valuable clues to the etiology of pellagra. Still more important would be some means by which an experimental pellagra could be induced in animals. This would enable us to do research on this disease, independently of pellagra districts.

The Results of our Knowledge of Deficiency Diseases Applied to Animal Metabolism and Nutrition.

The results of modern investigation of deficiency diseases seem to be unknown to most physiologists. I noticed only one *résumé*

which draws attention to the deficiency diseases with regard to general metabolism; this is the *résumé* of Mandel on protein metabolism [88]. The food was up to now valued only by its content in proteins, fats and carbohydrates, and calories value. From the time of the remarkable results of Emil Fischer on the chemistry of proteins a great number of different proteins of vegetable and animal origin were investigated. Each of the proteins was hydrolyzed and its content of amino-acids determined. The proteins themselves were used for metabolism experiments. It was shown that the proteins differ enormously in the quantity of their "building stones." The plant proteins especially, which are known to have an enormous content of glutaminic acid (up to 40 per cent.), differ from animal proteins, which contain on an average only 10 to 15 per cent. Some of these proteins, like gelatine, zein and gliadin, are known to be deficient in some of the amino-acids. Experiment has shown that proteins which are deficient in tyrosine, phenylalanine and tryptophane, are unable to keep the body in nitrogenous equilibrium. The relative quantities of amino-acids also constitute a very important factor. Michaud [89] has shown that by feeding dogs with their own proteins a relatively small amount of nitrogen suffices to keep the animals in nitrogenous equilibrium. Evidently an animal fed on proteins which differ in the quantity of amino-acids from the proteins of its own body is forced to use much more proteins and is unable to use these amino-acids, which are in larger proportion in the food than in its own body. The experiments of Abderhalden and Samuely [90], and Abderhalden, Funk and London [91], have shown that by feeding animals on gliadin, which contains 43 per cent. glutaminic acid, the composition of the proteins of the blood serum remains the same.

From this we can easily understand why Chittenden [92] finds that most people take in their food more nitrogen than is really necessary. In this food the organism is forced to find all the substances which are necessary for life, and which are present in this particular food in only small amount. By knowing all the substances which are necessary for life, it will be easy to choose a diet containing all those substances, and in this case the amount of nitrogen would probably be much below the figures obtained at present. At the same time this would solve the problem of choosing the cheapest food containing all the substances necessary

for life. We see from the present *résumé* that besides amino-acids small quantities of substances called vitamines must be contained in the food. Besides the vitamines described in this paper similar substances were found in milk. Osborne and Mandel [93], by feeding rats on different proteins, came to the conclusion that even proteins, which are probably complete in the number of amino-acids and contain the latter in a right proportion (casein), are capable of maintaining adult animals in nitrogenous equilibrium, but prevent normal growth in young animals. On adding to this diet milk freed from proteins the animals showed normal growth. Similar experiments were performed in this country by F. G. Hopkins.[1] I suppose that the substance facilitating growth found in milk is similar, if not identical, with the vitamines described by me.

In the metabolism experiments performed with the view of determining the nutritive value of different proteins the question of vitamines was not considered. We have seen, however, that animals kept on a diet deficient in vitamines lose enormously in weight, and do not maintain their nitrogen balance, even when the food is rich in nitrogen and calories. By an addition of small quantities of vitamines the animals kept on unchanged diet recover and are found to retain nitrogen. In future special care ought to be taken to supply the animal in metabolism experiments with sufficient quantities of the different vitamines. Only in this way can satisfactory and clear results be obtained. Abderhalden, in a recent paper [94], comes to the conclusion that the problem of artificial food is solved. Instead of synthetizing proteins it is possible to supply a mixture of amino-acids in the right proportion, and he thinks that all amino-acids and all necessary substances are already known. He fed dogs with meat which was broken up completely by ferments, and also with an artificial mixture of known amino-acids. His experiments were of short duration (seven days) and were performed after feeding for some time with broken-up meat, which contains, as was shown, all the protecting substances. An extension of his experiments for a longer period would undoubtedly result in his dogs getting scurvy. I agree with Abderhalden's general idea on this subject, but I think, however, that his conclusion is a little premature. The problem of artificial food cannot

[1] Private communication.

be solved, unless we know all the chemical constituents of our food. As has been shown in this paper, this is not the case, and many substances like vitamines await investigation; the synthesis of these substances will take many years of work still.

I suppose that the present *résumé* has already convinced the reader of the physiological importance of the vitamines. It is quite possible that their importance is much more considerable than is indicated in this paper. A predisposition to many other diseases may be induced by the deficiency of these protective substances. Among these diseases rickets may be mentioned. The investigation of this disease has not made much progress. Besides an abnormal calcium metabolism, which is probably the result and not the cause of the disease, nothing of importance is known. I think that experiments with vitamines, which can at least do no harm, ought to be performed here in order to ascertain if a deficiency of the latter is not the real primary cause of the disease. We will see the whole importance of these protective bodies when we inquire for what purpose these small quantities of substances are required in the animal organism. It is obvious that the minute amount necessary cannot be considered from the point of view of food. It is most probable that they are used as such or transformed into substances which are able to act in small quantities. Such substances in the body are known to be ferments, hormones and products of the secretion of internal glands. With the exception of adrenaline we know practically nothing about these substances. The secretions of thyroid and parathyroid glands, of pituitary and genital glands, are completely unknown and so are the enzymes and hormones. The further investigation of vitamines, the knowledge of their chemical composition, and their fate in the animal body, will probably help to elucidate these problems.

From the present *résumé* we can conclude that all the deficiency diseases can be prevented by a complete diet. A monotonous diet ought to be avoided, because in this case a deficient food is made use of for long periods and prepares the ground for the outbreak of the deficiency diseases. There is no doubt that as our knowledge of the relative value of different foodstuffs increases we will be able to prevent completely the outbreak of the latter.

REFERENCES.

[1] Sir Patrick Manson, "Tropical Diseases," 1900; "Handbuch der Tropenkrankheiten"; Miura, "Beri-beri," vol. ii, p. 140: "Traité de Path. exotique": Hébrard, "Le Béri-béri," vol. v, p. 1. [2] Wernich, "Geographisch-medizinische Studien.," Berlin, 1878. [3] van Leent, *Geneesk. Tydschr. voor Nederl. Indie*, 1880. [4] Takaki, *Sei-i-kwai*, 1885, 1886, and 1887. [5] Eykman, *Virchow's Arch.*, vol cxlix, p. 187, 1897. [6] Vordermann, *Geneesk. Tydschr. voor Nederl. Indie*, vol. xxxviii, 1898. [7] Braddon, "The Cause and Prevention of Beri-beri," London, 1907. [8] Fraser and Stanton, "Studies from the Institute for Medical Research, F.M.S.," No. 10, 1909. [9] Eykman, *Virchow's Arch.*, vol. cxlviii, p. 523, 1897. [10] *Idem.*, *Arch. f. Hyg.*, vol. lviii, p. 150, 1906. [11] Gryns, *Geneesk. Tydschr. voor Nederl. Indie*, vol. xli, 1901; vol xlix, 1909. [12] Bréaudat, *Bull. de la Soc. de Path. Exot.*, January, 1910; *Journ. de Chim. et Pharm.*, vol. vii, p. 447, 1911; Bréaudat et Dénier, *Ann. de l'Inst. Past.*, xxv, No. 2, 1911. [13] Fraser and Stanton, *Lancet*, p. 4515, 1910; "Studies from the Institute for Medical Research, F.M.S.," No. 12, 1911. [14] Schaumann, *Arch. f. Schiffs- und Tropenhyg.*, Beiheft v, p. 37, 1908. [15] Hulshoff Pol, *ibid.*, Beiheft iii, 1910. [16] Schaumann, *ibid.*, Beiheft viii, 1910. [17] Eykman, *ibid.*, Beiheft xv, p. 698, 1910. [18] Teruuchi, *Saikingakuzashi*, Tokio, No. 79, 1910. [19] Chamberlain and Vedder, *The Philippine Journal of Science*, vol. vi, June, 1911; vol. vi, p. 395, 1911. [20] Shiga and Kusama, *Arch. f. Schiffs- und Tropenhyg.*, Beiheft iii, 1911. [21] Aron and Hocson, *The Philippine Journal of Science*, vol. v, February, 1910. [22] Cooper and Casimir Funk, *Lancet*, p. 1267, November, 1911. [23] Kilbourne, *The Philippine Journal of Science*, vol. v, p. 127, 1910. [24] Kohlbrugge, *Cent. f. Bakt.*, 1 Abt., vol. lx, p. 223, 1911. [25] Casimir Funk, *Journ. of Physiol.*, vol. xliii, p. 395, 1911. [26] Schaumann, *Trans. of the Soc. of Trop. Med. and Hyg.*, vol. v, 1911. [27] Casimir Funk, *ibid.*, vol. v, p. 86, 1911. [28] *Idem.*, *Journ. of Physiol.*, vol. xliv, p. 50, 1912. [29] Palladino, *Biochem. Zeitschr.*, vol. xxxviii, p. 443, 1912. [30] Chamberlain, Bloombergh and Kilbourne, *The Philippine Journal of Science*, vol. vi, p. 177, 1911. [31] Fingerling, *Biochem. Zeitschr.*, vol. xxxviii, p. 448, 1912. [32] Greig, "Scientific Memoirs by Officers of the Medical and Sanitary Department of the Government of India," New Series, No. 45, 1911. [33] Holst, *Trans. of the Soc. of Trop. Med. and Hyg.*, vol. v, 1911. [34] Holst and Frölich, *Journ. of Hyg.*, vol. vii, p. 634, 1907; Holst, *ibid.*, p. 619. [35] Holst, *Verhandl. des 6 Nord. Kongress f. Innere Med.*, p. 328, 1909. [36] Fürst, *ibid.*, p. 349, 1909. [37] Nocht, "Festschr. zum 60 Geburtstage von R. Koch," p. 203, 1903. [38] Sir Thomas Barlow, *Medico-Chirurgical Trans.*, vol. xlvi, p. 187, 1883. [39] Fränkel, *Fortschr. auf dem. Gebiete der Röntgenstrahlen* vol. vii, 1904; vol. x, 1906. [40] Looser, *Jahrb. f. Kinderheilk.*, December, 1905. [41] Vortisch van Vloten, *Arch. f. Schiffs- und Tropenhyg.*, vol. xv., p. 380, 1911. [42] Neumann, *Deutsch. med. Woch.*, vol. xxviii, pp. 628, 647, 1902. [43] Heubner, *ibid.*, vol. xxix; *Vereinsbeilage*, pp. 109, 110, 117, 1903. [44] A. Meyer, "Barlow's Disease," Copenhagen, 1901. [45] Brachi and Carr, *Lancet*, p. 662, 1911. [46] Bordas and Raczkowski, *Comptes rendus de l'Acad. des Sciences*, vol. cxxxvi, p. 56, 1903. [47] Bartenstein, *Jahrb. f. Kinderheilk.*, vol xi, p. 6, 1905. [48 Frölich, *Verh. des 6 Nord. Kongress*

f. Innere Med., 1909. [49] Stepp, *Biochem. Zeitschr.*, vol. xxii, p. 452, 1909; *Zeitschr. f. Biol.*, vol. lvii, p. 135, 1911. [50] Delpech, *Ann. d'hyg. publique*, vol. xxxv, 1871. [51] Bucquoy, *Union médicale*, September, October, 1871. [52] Hodson, *Lancet*, p. 1037, 1910. [53] Ceni, *Ziegler's Beitr.*, vol. xxxix. [54] Otto, *Zeitschr. f. klin. Med.*, vol. lix, Hefte 2-4. [55] Lombroso, "La Pellagra," Torino, 1892. [56] Gosio, *Riv. med.*, 1893; *Riv. pellagr. ital.*, No. 3, 1903. [57] Gavina, *Riv. pellagr. ital.*, vols. vi. and viii. [58] Bertarelli, *Centr. f. Bakt.*, 1 Abt., vol. xxxiv, p. 34, 104, 1904. [59] Antonini, "La Pellagra." Milano, 1902. [60] Camurri. *Cent. f. Bakt.*, 1 Abt., vol. liii, p. 438, 1910. [61] v. Neusser, *Münch. med. Woch.*, 1887. [62] de Giaxa, *Ann. d'Ig. sperim*, vol. ii, No. 8, 1892. [63] di Pietro, *ibid.*, No. 2, 1902. [64] Ceni, *Riv. sperim. di freniatria*, vols. xxvii. and xxix. [65] Tizzoni, *Cent. f. Bakt.*, 1 Abt., vol. xlvi, p. 310, 1908. [66] Tizzoni and Panichi, *ibid.*, vol. xliv, p. 210, 1907. [67] Sambon, *Journ. of Trop. Med. and Hyg.*, vol. xiii, pp. 271, 287, 305, 319, 1910. [68] Roberts, *Journ. of the Amer. Med. Assoc.*, p. 1713, 1911. [69] British Pellagra Commission, *Journ. of Trop. Med. and Hyg.*, vol. xiv, p. 374. 1911. [70] Babès and Vasiliu, *Berl. klin. Woch.*, vol. xliv, p. 1189, 1907. [71] Babès, Vasiliu and Gheorghus, *ibid.*, vol. xlvi, p. 237, 1909. [72] Babès and Busila, *Comptes rendus de la Soc. de Biol.*, vol. lxx, p. 602, 1911. [73] Tizzoni, *Cent. f. Bakt.*, 1 Abt., vol. lxi, p. 403, 1911. [74] Alessandrini, *Policlinico Seria pratica*, 1910. [75] Nevius-Hyde, *Amer. Journ. of the Med. Sciences*, January, 1910. [76] Clarke, Hamill, Pollock, Curtis and Dick, *Journ. of Infectious Diseases*, vol. x, p. 186, 1912. [77] Raubitschek, *Wien. klin. Woch.*, vol. xxiii, p. 963, 1910; *Cent. f. Bakt.*, 1 Abt., vol. lvii, p. 193, 1911. [78] Aschoff, "Handb. der allg. Path. von Krehl und Marchand," vol. i, p. 159. [79] Hausmann, *Wien. klin. Woch.*, vol. xxiii, p. 1287, 1910; *Biochem. Zeitschr.*, vol. xiv, p. 275, 1908; vol. xv, p. 12, 1909. [80] Horbaczewski, *Oesterr. Sanitätswesen Beilage*, No. 31, August, 1910. [81] Lode, *Wien. klin. Woch.*, No. 31, August, 1910. [82] Lavinder, *Public Health Reports*, May, 1911. [83] Babès, *Intern. Cong. of Path.*, Turin, 1911. [84] Hausmann, *Oesterr. Sanitätswesen*, 1911. [85] Bezzola, *Zeitschr. f. Hyg.*, vol. lvi, p. 75, 1907. [86] Lucksch, *ibid.*, vol. lviii, p. 474, 1908. [87] Holst, *Medicinsk Revue Festschrift*, July, 1911. [88] Mandel, *Ergebn. der Physiol.*, vol. xi, p. 418, 1911. [89] Michaud, *Zeitschr. f. physiol.*, ch. lix, p. 405, 1909. [90] Abderhalden and Samuely, *ibid.*, vol. xlvi, p. 193, 1906. [91] Abderhalden, Casimir Funk and London, *ibid.*, vol. li, p. 269, 1907. [92] Chittenden, "The Nutrition of Man." New York, 1907. [93] Osborne and Mandel, *Science*, New Series, vol. xxxiv, p. 722, 1911. [94] Abderhalden, *Zeitschr. f. Physiol.*, ch. lxxvii, p. 22, 1912.

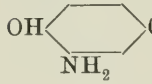
John Bale, Sons & Danielsson, Ltd., 83-91. Great Titchfield Street, London, W.

CIV.— *The Constitution of Aminotyrosine and the Action of Oxydases on Some Tyrosine Derivatives.*

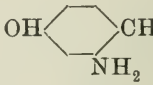
By Casimir Funk.

In connexion with the synthesis of *dl*-3:4-dihydroxyphenylalanine (Trans., 1911, **99**, 554), an attempt was made to obtain the optically active compound by employing aminotyrosine, which was considered by Neuberg to be 3-aminotyrosine ("Handbuch der Biochemie," Oppenheimer, Vol. 4, Part II., p. 363). An analogous process was used by Erlenmeyer and Lipp (*Annalen*, 1883, **219**, 166) in the preparation of tyrosine from aminophenylalanine. Aminotyrosine was prepared according to the methods described by Strecker (*Annalen*, 1850, **73**, 70), Staedeler (*ibid.*, 1860, **116**, 57), and Beyer (*Zeitsch. Chem.*, 1867, 437) from tyrosine through the nitro-compound. In these papers very few experimental data, no melting points, reactions, or analyses were given. The tyrosine employed for the synthesis was optically active *l*-tyrosine, but both the nitro- and amino-tyrosine obtained were completely inactive, so that racemisation must have occurred during the preparation of the nitro-compound. By diazotising the aminotyrosine according to Erlenmeyer and Lipp's method, no 3:4-dihydroxyphenylalanine was obtained, but a substance was isolated which possessed the

composition of the initial product, differing, however, from the latter by its resistance to oxidation, and especially to the action of oxydases. Bertrand (*Bull. Soc. chim.*, 1896, [iii], **15**, 791) has shown that these ferments are only able to oxidise ortho- and para-compounds. It must be concluded, therefore, that the new non-oxidisable aminotyrosine is a meta-compound, and that the original nitro- and amino-tyrosine are mixtures of two isomerides, namely, the ortho- and meta-compounds:



and



By the action of nitrous acid, 3-aminotyrosine is destroyed and 2-aminotyrosine, described above, left in the solution.

Until recent times the belief was current that the ferments laccase and tyrosinase were specific in their action; thus laccase was considered to oxidise only phenols and aminophenols, tyrosinase only tyrosine. It has, however, since been shown that this is not the case, and in the present paper further proof of this is given, and it is shown that 3:4-dihydroxyphenylalanine, aminotyrosine, and adrenaline are oxidised equally well by both ferments.

The action of laccase and tyrosinase might be used with good results for the determination of the position of hydroxy- and amino-groups in compounds, analogous to those described in this paper.

Experimental.

Preparation of Nitrotyrosine.

l-Tyrosine (150 grams) from silk was suspended in water, and nitric acid (600 c.c.; D 1·3) was added drop by drop, the mixture being kept cool in cold water and continually stirred with a turbine. During the reaction the nitrate of the nitrotyrosine separated in golden-yellow needles (130 grams), which melted and decomposed at 170°. The crystals were dissolved in a little water, ammonia was added, and the solution was evaporated on the water-bath until crystals began to separate. Nitrotyrosine was thus obtained in yellow needles, which after drying under diminished pressure at 110°, melted at 220°, and were optically inactive. Yield, 104 grams.

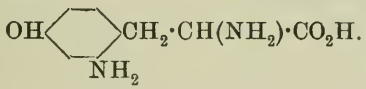
Preparation of Aminotyrosine.

Nitrotyrosine (104 grams) was dissolved in water and heated on the water-bath for one hour with tin (200 grams) and hydrochloric acid (1200 c.c.; 4:10). The warm solution was then decanted, freed from tin by means of hydrogen sulphide, and the filtrate evaporated under diminished pressure in a stream of coal-gas. Colourless needles of dichloroaminotyrosine separated out, which melted at 155—160°. The hydrochloride was dissolved in a very little water, and neutralised with the calculated amount of 2*N*-potassium hydroxide. On evaporation under diminished pressure, aminotyrosine (60 grams) was obtained, which on recrystallisation from water gave slightly grey-coloured prisms melting and decomposing at 265°. This compound gave with ferric chloride a pink coloration, and with Millon's reagent first an orange coloration, followed by reduction of the mercury salt. The aqueous solution assumes a pink colour after a short time. The substance is optically inactive:

0·233, by Kjeldahl's method, required 23·6 c.c. *N*/10-H_2SO_4. N = 14·18.

$C_9H_{12}O_3N_2$ requires N = 14·28 per cent.

Preparation of 2-Aminotyrosine,



A solution of aminotyrosine (10 grams in molecular proportions of sulphuric acid) kept at −2° was treated with a slow stream of gaseous nitrous acid, prepared from 3·6 grams of sodium nitrite dissolved in 50 c.c. of water and 5·1 grams of sulphuric acid in 50 c.c. of water. The solution was then boiled for one hour, during which time a little nitrogen was evolved. After the removal of sulphuric acid by means of barium hydroxide, the solution was evaporated under diminished pressure. The residue yielded 5 grams of yellow needles, which after recrystallisation from water and drying under diminished pressure at 110° melted at 273°. With ferric chloride a pink coloration and with Millon's reagent a white precipitate were obtained. The aqueous solution was not oxidised on contact with air:

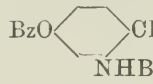
0·2074 gave 0·4188 CO_2 and 0·1122 H_2O. C = 55·07; H = 6·01.

0·1868, by Kjeldahl's method, required 18·6 c.c. *N*/10-H_2SO_4. N = 13·99.

$C_9H_{12}O_3N_2$ requires C = 55·06; H = 6·17; N = 14·28 per cent.

This substance is aminotyrosine, and differs from the initial product by the melting point, stability against oxidation, the colour of the crystals, and, as is shown later, in its behaviour towards oxydases. From the latter point of view the substance must be considered to be *2-aminotyrosine*. The mixture of both substances melted at 260°.

Tribenzoyl-2-aminotyrosine,



Aminotyrosine (0·5 gram) was dissolved in *N*-potassium hydroxide (5·1 c.c.); to this solution sodium hydrogen carbonate (3·5 grams) was added, and then benzoyl chloride (2·3 grams) in small portions at a time. The compound separated out during the reaction as the sodium salt. It was then treated with dilute sulphuric acid, the precipitate finally extracted with light petroleum to dissolve the benzoic acid, and recrystallised from a mixture of acetic acid and ethyl acetate. Colourless, microscopical needles (0·9 gram) were obtained, which were sparingly soluble in alcohol or ethyl acetate, readily so in acetic acid, and insoluble in water. After drying under diminished pressure at 100°, they melted and decomposed at 192—195°:

0·1766 gave 0·4602 CO_2 and 0·0746 H_2O. C = 71·07; H = 4·69.

$C_{30}H_{24}O_6N_2$ requires C = 70·83; H = 4·76 per cent.

An attempt was then made to separate the mixture in the initial product by means of benzoylation, but was unsuccessful. Three grams of the initial aminotyrosine yielded only 0·3 gram of a benzoyl compound which melted at 191—195°, whilst in the mother liquor no further product could be detected. By mixing both benzoyl compounds, the melting point was not depressed.

Action of Laccase and Tyrosinase.

For these experiments 0·05 gram of each substance was dissolved in 5 c.c. of water, and 1 c.c. of the ferment solution added. The tyrosinase was prepared from *Agaricus campestris*, and the laccase from the latex tree (*Rhus succedanea*). The colorations were

	Laccase.	Tyrosinase.
Quinol	red	no change
Tyrosine	no change	black
Aminotyrosine	black	black
2-Aminotyrosine	no change	no change
3 : 4-Dihydroxyphenylalanine	pink	pink
dl-Adrenaline	pink	pink
Nitrotyrosine	no change	no change

observed after five minutes. The experiments with quinol and tyrosine showed that the laccase was free from tyrosinase and the tyrosinase free from laccase.

The difference between the aminotyrosines is thus evident. These experiments also reveal the new fact that many substances are oxidised both by laccase and tyrosinase.

LISTER INSTITUTE OF PREVENTIVE MEDICINE,
CHELSEA S.W.

R. CLAY AND SONS, LTD., BRUNSWICK ST., STAMFORD ST., S.E., AND BUNGAY, SUFFOLK.

[From THE JOURNAL OF HYGIENE, Vol. XII, No. 2, June 19, 1912.]

OCCURRENCE OF FERMENTS IN THE STERILE MILK COLLECTED BY MILKING TUBE FROM COWS AND GOATS.

By ARTHUR HARDEN
and JANET E. LANE-CLAYPON.

(*Biochemical Department, Lister Institute.*)

The literature upon the presence of various ferments in milk is extremely large and dates back through many years. Up to the beginning of 1911, however, when the experiments described in this paper were undertaken, no observer had dealt with milk uncontaminated by bacteria.

Many observers had collected milk with very great care, but it did not appear that anyone had obtained sterile milk for the purpose of investigation of the ferments, although the method of obtaining sterile milk had been described by Lister (1878) and frequently employed for various purposes. It was decided to adopt the method described by Swithinbank and Newman (1903), for the collection of sterile milk, by means of a milking-tube or catheter.

The apparatus used by these observers consists essentially of two portions; the first is a milking-tube connected by about 3 or 4 ft. of rubber-tubing to a piece of glass tubing. The milking-tube and glass tube are both enclosed in test-tubes the open ends of which are blocked by plugs of cotton wool, so that the whole can be sterilised and preserved sterile.

The second part of the apparatus is a filter flask, through the cork of which passes a piece of glass tubing, closed by a short piece of rubber-tubing, and a clamp. Both the end of the rubber-tubing and the side tube of the flask are plugged with cotton wool, so that this also can be kept sterile.

The teat of the cow's udder which is to be used is carefully cleaned with 2 % lysol, as are also the hands of the experimenter. A little

milk must be milked away so as to remove that portion of the milk which had been stagnating in the mouth of the teat. The milking-tube is now removed from the test-tube and inserted into the teat, when the milk flows into the test-tube at the other end of the rubber-tubing. As a rule the milk runs freely but occasionally the udder has to be "milked" a little, as the cow "holds the milk back."

The milk which flows into the test-tube should be discarded or used for boiled controls. The rubber-tube is now disconnected from the glass tube, and (the cap of india-rubber having been removed from the tube of the filter flask) connected to the filter flask, so that the milk runs into the flask, and the requisite quantity can be collected.

This apparatus was used throughout the experiments, with satisfactory results. While the work was in progress, a paper by Rullmann (1911) appeared, covering the ground upon which we were working, the results obtained by him agreeing in almost every particular with those which we were obtaining.

We decided however to continue the experiments, especially as the method which we were employing for the estimation of catalase was different from that used by him.

Both goats' and cows' milk were used, the goats being kept on the premises, and thus easily accessible. The cows' milk was obtained from a neighbouring dairy where cows were kept under good conditions. We are much indebted to Mr E. J. Walker the owner of the dairy for allowing us to use his cows for the purpose of these experiments and desire to take this opportunity of thanking him for his kindness.

Experiments upon goats' milk.

The ordinary milking-tube used for cows was found to be rather too large for the goat's teat and a smaller size of silver catheter was obtained. Immediately after collection plates were made of the milk. The amount taken was either 1 or 2 c.c. and the medium used was nutrient agar, and also whey-litmus-agar. Some of the plates were incubated at 37° C. and others at 22° C. for four or five days.

In no case did we succeed in getting all the plates free from any growth, although not infrequently some of the plates remained sterile. The count varied from 0·3–9·7 per c.c., the organisms being for the most part moulds which had evidently obtained entrance from the outside air, and possibly during the process of plating. In a few cases the goats were difficult to catheterise, and kicked out the catheter, the slight

contamination observed being probably introduced in this way. The ferments investigated were peroxidase, catalase and both direct and indirect reductase (Schardinger, 1902), and the results obtained for each ferment are dealt with separately.

Peroxidase.

Para-phenylene-diamine and hydrogen peroxide were used for the detection of this ferment, 1 c.c. of a 1 % solution of the para-phenylene-diamine, and 0·4 c.c. of a 1 % solution of the peroxide being employed. The reaction was in all cases instantly positive, the controls with boiled milk being always negative. It seems clear therefore that goats' milk while in the udder contains peroxidase.

Catalase.

In the first three experiments an Einhorn Saccharimeter was used, which is practically the method known as Koning's (Koning, 1906), and was that used by Rullmann in his experiments with cows' milk.

The results obtained are given below. The amounts used were 15 c.c. milk, 1 c.c. water and 5 c.c. of 1 % H_2O_2 incubated at 37° C. The water was used to make sufficient fluid in the saccharimeter.

	Method of procuring milk	Bacterial count per c.c.	Amount of oxygen given off in c.c.	Control
1.	Taken by catheter	5·0	In 45 mins. = 0. In 1½ hrs. 0·25. In 17 hrs. = 0·5	Negative.
2.	Milked into sterile flask	350·0	In 3 hrs. = 0. In 21 hrs. = 0·25	,,
3 *a*.	Milked into sterile flask	Plates spoiled by sporing	In 3 hrs. = 1 c.c. In 4 hrs. = 1·2 c.c.	,,
3 *b*.	Taken by catheter	3·0	In 1 hr. = 1 c.c. In 4 hrs. + 1 c.c.	,,

These experiments showed that the Einhorn saccharimeter was not reliable for the estimation of the gas evolved, owing to the impossibility of shaking the apparatus. The importance of shaking in the estimation of catalase has been pointed out by many observers, and was brought prominently into discussion in the papers published in 1911 by Kooper (1911) and Grimmer (1911). The Lobeck apparatus was also tried but it was finally decided to use the apparatus devised by Harden, Thompson and Young (1910) in estimating the gas evolved in

alcoholic fermentation. This consists of a flask with long neck, capable of containing about 100 c.c. of fluid, which is clamped into a water-bath of desired temperature, and connected with rubber and glass tubing to a eudiometer, so that it can be taken out of the water-bath and shaken, without any disturbance of the gas content of the apparatus. The apparatus is so arranged that the pressure on the gas in the flask is always equal to that of the atmosphere.

In these experiments 50 c.c. of milk and 5 c.c. H_2O_2 (1 %) were used throughout both for goats' and cows' milk, control experiments with H_2O_2 having shown that this quantity of H_2O_2 gave the optimum evolution of gas with 50 c.c. milk at 25° C., the temperature at which the water-bath was kept.

5 c.c. of a 1 % solution of hydrogen peroxide are capable of evolving about 18 c.c. of oxygen at atmospheric temperature and pressure under the influence of catalase and hence, as soon as this number was approached, a fresh quantity of H_2O_2 was added.

All the apparatus with which the milk came in contact was sterilised. After the 50 c.c. of milk had been pipetted into the flasks they were allowed to remain for at least 10 minutes in the water-bath before the hydrogen peroxide was added. The eudiometer was then adjusted, the flasks shaken and a reading taken every 5 minutes.

The following were characteristic experiments and results:

I. Bacterial count 1 per c.c.

3.40 p.m. 5 c.c. H_2O_2 added, by 5.17 p.m.	12·6 c.c. O_2 given off.
5.20 p.m. 5 c.c. more added, by 6.20 p.m.	14·1 c.c. O_2 given off
6.20 p.m. 5 c.c. more added, by 7.0 p.m.	8·6 c.c. O_2 given off.
In 2 hours 40 minutes	35·3 c.c. O_2 in all.

II. Bacterial count 1·6 per c.c.

2.18 p.m. 5 c.c. H_2O_2 added, by 2.39 p.m.	12·8 c.c. O_2 given off.
2.46 p.m. 5 c.c. more added, by 3·6 p.m.	14·1 c.c. O_2 given off.
3.12 p.m. 5 c.c. more added, by 4.40 p.m.	18·0 c.c. O_2 given off.
In 2 hours 22 minutes	44·9 c.c. in all.

The controls with boiled milk in each case were negative.

These results show that a much higher figure is obtained when the shaking is carried out at short intervals during the experiment, than when the Einhorn tube is used, and they leave no doubt that catalase is present in the milk of goats as it leaves the gland.

Reductase.

The reduction of methylene blue was tested for, both with methylene blue alone and also with Schardinger's reagent, the tubes being incubated at 37° C. Ten c.c. of milk were taken, and 0·1 c.c. of the reagent. In the milk collected by catheter there was no reduction of methylene blue alone in any of the tubes, the times of observation varying from 1½ to 18 hours. The only case in which reduction occurred was in milk which was milked by the animal attendant, and had an initial bacterial count of 350 per c.c. This milk reduced the methylene blue in two hours completely.

Schardinger's reagent was not reduced on any occasion except that upon which there was reduction of the methylene blue. This however was probably merely a reduction of the methylene blue and not a specific action on the Schardinger's reagent. It seems therefore that there is no reductase either direct or indirect present in sterile milk from goats.

Koning (1906, 1907), working with carefully collected goats' milk used as soon as possible after milking, obtained similar results, although he considered that there must be traces of reductase present, since, when goats' milk was added to cows' milk, the reduction of Schardinger's reagent took place more rapidly than when the cows' milk was simply diluted with water. Sames (1910) however has found that Schardinger's aldehyde-reductase will not bear dilution, so that if this be the case Koning's results might really show a delay in the action of the ferment in the cows' milk as a result of dilution by water and not an acceleration of the action by the addition of the goats' milk.

Experiments with cows' milk.

The same method as was used for the detection of the ferments in goats' milk was used for the cows' milk. The milk was collected by catheter, and brought at once to the Lister Institute, the several reactions being started as soon as possible after arrival.

Peroxidase.

The bacterial counts of the specimens of milk used for the peroxidase reactions varied from 0–6·0 per c.c. The reaction was strongly positive on all occasions. On one occasion when the cow had calved 2–3 weeks before the milk was examined, the reaction with para-phenylene-diamine was positive before the addition of the peroxide,

indicating the presence of an oxidase. Inasmuch as the oxidases have been shown to be a mixture of a peroxide and of a peroxidase this observation has no special significance for the present work. Rullmann also found peroxidase constantly present in sterile milk from cows.

Catalase.

The reaction in this case was always positive, as it was also in Rullmann's work, but the amount of O_2 given off was frequently very small, and much less than that given off by the goats' milk.

The following are some of the experiments; in all cases 50 c.c. of milk were taken. Controls of boiled milk in all instances gave negative results.

I. Bacterial count 0.

11.55 a.m. 5 c.c. H_2O_2 added, and flask shaken every 5 minutes.
12.55 p.m. 2·5 c.c. O_2 given off, 5 c.c. more H_2O_2 added.
3.45 p.m. 1·2 c.c. O_2 given off.

3·7 c.c. in all.

In 3 hours 50 minutes 3·7 c.c. O_2 given off from 50 c.c. milk.

II. This experiment was carried out in the severest heat of summer (1911), and unavoidably towards midday. The cow in this case kicked out the catheter, and the bacterial count was 47 per c.c. 6·0 c.c. were given off in 3 hours 25 minutes. It is unlikely that the rather higher amount of catalase was due to the action of the bacteria, since, even at the end of the three hours, the number present cannot have been high, and in subsequent experiments with milk practically free from organisms equally high values were obtained.

III. Bacterial count 2·0 per c.c.

In ½ hour 6·3 c.c. O_2 were evolved from 50 c.c. whole milk.
In 4 hours 12·6 ,, ,, ,, ,, ,,
In ½ hour 1·2 ,, ,, ,, 45 c.c. skimmed milk.
In 4 hours 2·8 ,, ,, ,, ,, ,,
In ½ hour 2·5 ,, ,, ,, cream of 45 c.c. of milk.
In 4 hours 4·5 ,, ,, ,, ,, ,, ,,

The cream was made up to 45 c.c. with sterilised saline.

IV. Bacterial count about 6 per c.c. Plates spoiled by sporing organisms.

In 50 mins. 5·9 c.c. O_2 were evolved from 50 c.c. whole milk.
,, 2·4 ,, ,, ,, 45 c.c. skimmed milk without sediment.
,, 0·9 ,, ,, ,, cream from 45 c.c. of milk.

Experiments upon centrifugalised milk as well as upon whole milk were also carried out, with the following results.

V. Bacterial count 1 per c.c.

In ½ hour 3·7 c.c. O_2 were given off by whole milk.
„ 2·9 „ „ „ whole milk without sediment.
„ 0·4 „ „ „ the sediment alone.

The sediment was examined for leucocytes of which only a few were present. This experiment shows that the seat of the catalase is not wholly or even chiefly in the leucocytes of the sediment, as was also found by Meyer. Jensen, Torday and others found the catalase to be associated with the fat content, and Jensen also found that the strippings contained more catalase and more leucocytes than the rest of the milk, but that the amount of catalase was very slight compared with that produced by the bacteria.

Catalase therefore is present in cows' milk collected by catheter, having a negligible bacterial content; the amount present being variable.

This agrees with the work of Rullmann except that our figures are higher than his, but this is probably accounted for by the different apparatus used, for Rullmann used the Koning apparatus which as was seen in the case of the goats' milk gave lower figures than that employed by us.

Rullmann's figures for 10 c.c. milk were as follows:

In 1 hour from 0·0—traces of O_2.
In 12–18 hours 0·2–1·8 c.c. O_2.
In 24 hours 0·2–3·6 c.c. O_2.

Reductase.

The reduction of methylene blue was negative in every case, even when the observations extended over more than 18 hours at 37°. With Schardinger's reagent the reaction was very variable.

I. Bacterial count 0 per c.c. Positive in 3 hours at 37° C.
II. Bacterial count 47 per c.c. Positive in 5 hours at 37° C.
III. Bacterial count 47 per c.c. Negative after 2 hours 45 mins. at 37° C.
IV. Bacterial count about 13 per c.c. Positive in 3¼ hours at 37° C.
V. Bacterial count 2 per c.c. Positive in 2¼ hours at 37° C.
VI. Bacterial count 1 per c.c. Negative after 28 hours at 37° C.

The irregularity of the reaction is probably to be explained by the fact that sometimes the cows had been fairly recently milked and at other times not. Jensen (1906) and Romer and Sames (1910) have shown that the strippings have the highest reducing value for

Schardinger's reagent, and the last two authors have further shown that the first milk rarely contains any aldehyde-reductase (the ferment reducing Schardinger's reagent).

Reinhardt and Seibold (1911) have also shown that the reaction with Schardinger's reagent is often negative during the first two months after calving, and that the time of its appearance is also very irregular.

No precise data could be obtained in regard to the time after calving of the cows from which the milk was obtained, and these facts are sufficient to account for the variable results obtained. The length of time taken for the reaction to develop is longer than that observed by most investigators but the optimum temperature is from 45°–50° C. and the foregoing observations were made at 37° C. which accounts for this point of difference. Rullmann also found direct reductase absent and the reaction with Schardinger's reagent positive, in catheter milk.

Conclusions.

1. The presence of peroxidase and catalase can be demonstrated in the milk obtained by catheter from both goats and cows.

2. The catalase content of goats' milk is apparently higher than that of cows' milk.

3. The reduction of methylene blue does not occur with catheter milk of either goats or cows, at any rate within many hours.

4. Schardinger's reagent is not reduced by goats' milk, but catheter milk from a cow frequently reduces it.

REFERENCES.

Grimmer (1911). *Milchwirtschaftliches Zentralbl.* vii. 314.
Harden, Thompson and Young (1910). *Biochem. Journ.* v. 230.
Jensen (1906). *Révue générale du lait*, vi. 56, 85.
Koning (1906). *Milchwirtschaftliches Zentralbl.* ii. 517.
—— (1907). *Ibid.* iii. 41.
Kooper (1911). *Ibid.* vii. 264, 411.
Lister (1878). *Collected Papers*, i. 364.
Meyer, J. (1910). *Arb. a. d. Kaiserl. Gesundheitsamte*, xxxiv. 118–121.
Reinhardt and Seibold (1911). *Biochem. Zeitschr.* xxxi. 295.
Romer and Sames (1910). *Zeitschr. f. Unters. Nahr. u. Genussm.* xx. 1.
Rullmann (1911). *Arch. f. Hyg.* lxxiii. 81.
Sames (1910). *Milchw. Centralbl.* vi. 462.
Schardinger (1902). *Zeitschr. f. Unters. Nahr. u. Genussm.* v. 1113.
Swithinbank and Newman (1903). *Bacteriology of Milk*, p. 42.
Torday, E. and Torday, A. (1908). *Jahrb. f. Kinderh.* lxvii. 277.

[*Reprinted from the Journal of Physiology,*
Vol. XLIII. No. 1, *September* 11, 1911.]

THE OXIDATION OF ISOLATED ANIMAL TISSUES.

By ARTHUR HARDEN and HUGH MACLEAN.

(*From the Lister Institute. Biochemical Department.*)

In the course of a former investigation(1), on the alleged post-mortem occurrence of alcoholic fermentation in animal tissues, it was found that no apparent formation of carbon dioxide from sugar was obtained when the experiments were conducted in an atmosphere of carbon dioxide or hydrogen. Since, however, the living tissues contain normally a considerable amount of oxygen, it was thought advisable to determine whether any evidence of glycolysis of this nature could be found when the experiments were conducted in an atmosphere of oxygen. In carrying out this plan it was necessary to determine the normal gaseous metabolism of the tissues, since it is well known that oxygen continues to be absorbed and carbon dioxide given out for a considerable time after death. A number of researches on this subject have been carried out by former investigators, but a perusal of the literature shows that the results obtained by different authors vary to such a degree as to render them useless for practical application; the very great variations in the results recorded indicated that no reliance could be placed on the averages of these figures, and it was therefore found necessary to investigate the subject from the beginning.

As will be seen the figures obtained in this investigation differ very considerably from those given in many of the researches already published, but on the whole they are fairly consistent, and as the apparatus employed is capable of recording accurately differences of 1 c.c. of gas, it may fairly be claimed that under the conditions of the experiments the results indicate with a considerable degree of accuracy what actually takes place.

The most elaborate and recent investigation on this subject has been carried out by Battelli and Stern(2), but their results vary in an extraordinary manner, and it is extremely doubtful whether the method they adopted is sufficiently accurate. Unfortunately their description

of the apparatus employed is so lacking in details as to render it very difficult to understand the exact manner in which their results were obtained.

As no very satisfactory method is described we found it necessary to construct an apparatus by which an accurate determination of the amount of oxygen absorbed and the carbon dioxide given out could be obtained. After several preliminary experiments it was found that the following arrangement answered exceedingly well, and after a little practice very accurate readings could be obtained.

Description of apparatus. The apparatus employed is a modification and elaboration of a form previously used by Thompson in this laboratory, for estimating the gaseous metabolism of micro-organisms(3). An arrangement was adopted whereby a continuous stream of oxygen could be circulated through the apparatus. The whole was closed and air-tight, and at one point in the circulation a vessel containing strong standard potash was introduced; at another point was placed a flask containing the minced up organ. The oxygen passed through the minced tissue and afterwards through the potash, so that any carbon dioxide derived from the tissue was immediately absorbed by the alkali, and could subsequently be estimated. Any oxygen absorbed by the tissue was indicated by a scale on a burette so that the amount of carbon dioxide given out and the amount of oxygen absorbed in any given time could be readily determined.

The details of the apparatus are as follows:

Two ordinary nitrometers, *A* and *B* (Fig. 1), were taken and to the upper end of each a two-way tap (*C* and *D*) was attached; in the photograph given below (Fig. 2) these taps are sealed on, but the attachment can be more easily made by means of rubber stoppers (the two lower taps seen in the photograph belong to the nitrometer and are not necessary for the purpose of this apparatus). The nitrometers are connected by rubber tubing with reservoirs filled with mercury. Another two-way tap (*E*, Fig. 1) is seen on the left of the sketch; this is connected with the potash flask by the tube *F*, while on the lower side it opens into nitrometer *A* or *B*, according to the position of the tap.

A three-way tap *H* is connected with both nitrometers and through another three-way tap *K* with the flask in which the tissue is placed, or with the air, as desired. When the taps are in the position represented in the diagram, and the reservoir of *B* raised, it will be seen that there is direct communication between nitrometer *B* and the flask containing the minced organ (through *H* and *K*), so that oxygen passing through

from *B* would pass up along the unshaded tubes through the flask, afterwards passing through the potash bottle and along tube *F* to nitrometer *A*. By reversing the positions of the reservoirs and of all the taps except *K*, oxygen would pass from nitrometer *A* along the shaded path to *H*, then round the circuit as before to tap *E*, and finally along the shaded path to nitrometer *B*. In this way by alternately raising and lowering the mercury bulbs and reversing the taps a continuous circulation could be kept up. The photograph shows the actual apparatus used in the experiments. When an experiment is to be made the apparatus is connected by means of a rubber tube with a cylinder containing oxygen. Then tap *K* is turned so as to allow oxygen to pass along to the tissue and potash flasks, and back to tap *E*, which is removed; when sufficient oxygen has been passed through, tap *E* is replaced and taps *K* and *H* opened towards one of the nitrometers.

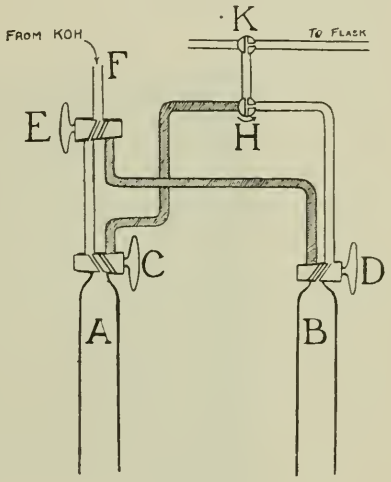


Fig. 1.

The mercury reservoir of this nitrometer is now lowered, and the nitrometer filled with oxygen to the desired level. When sufficient oxygen is passed, tap *K* is turned round to connect the flask with tap *H*. In the photograph (Fig. 2) nitrometer *B* is seen partly filled with oxygen, while *A* is filled with mercury. Both nitrometers are graduated in fifths of a cubic centimetre. The flask for the tissue to be investigated is placed in a water bath kept at 37° C. and is connected with the circulatory apparatus by means of a tight rubber stopper through which pass an inlet and outlet tube. A weighed amount of substance is now quickly placed in the flask, some more oxygen passed in, and the stopper tightly replaced. To allow for changes caused by differences of temperature the whole is left for ten minutes, and then oxygen is circulated until a constant reading[1] is obtained. This gener-

[1] In connection with this point, a *constant* reading is obtained only in cases where oxidation is slow. At the beginning of the experiment the expansion of oxygen gives an increase in the readings, and when this passes over there is a steady decrease in proportion to the activity of the tissue. Equilibrium is therefore considered to be attained when either constant or steadily decreasing readings are obtained.

ally means that oxygen has to be circulated about fifteen to twenty times, and occupies about five minutes. After a constant reading is obtained the flask is removed from the bath at frequent intervals and shaken vigorously. At the end of the experiment oxygen is again circulated till a constant reading is obtained, while during the experiment it is also circulated at frequent intervals in order to prevent any possible accumulation of carbon dioxide and give the maximum chance

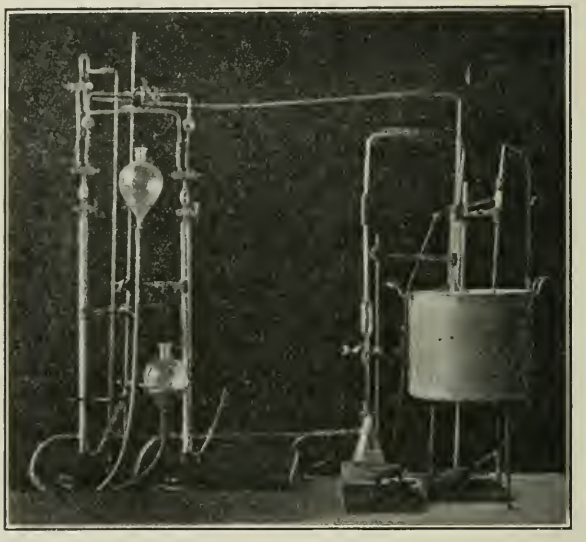


Fig. 2.

for oxidation to occur. The difference between the first and final constant readings gives the amount of oxygen absorbed by the tissues, whilst titration of the caustic potash indicates the amount of carbon dioxide given off. Though the apparatus seems a little complicated it is really not so in practice; it is easily fitted up and works well.

As there was a tendency for some mercury to pass up the smaller tubes, small bulbs with traps were employed to overcome this difficulty. They are seen in the photograph immediately above the taps corresponding to *C* and *D* of Fig. 1.

Experimental.

In the first experiments carried out an attempt was made to determine the amount of carbon dioxide given off as the result of oxidation by the absorbed oxygen. It is well known that animal tissues, even in the absence of oxygen, are capable of giving off considerable amounts of carbon dioxide under the conditions of the experiment, but especially when boiled for some time with weak acids. Pflüger(4) and Stintzing(5) obtained very large amounts of carbon dioxide from tissues treated in the latter manner; in many cases 100 gms. of rabbit muscle yielded over 100 c.c. In our experiments however we failed to obtain such large quantities though the method adopted was in many cases practically the same as that used by the above observers. The tissue was taken as soon as possible after death, minced up very quickly, washed, and placed in a flask; some acidified water was then added and the mixture boiled for $1\frac{1}{2}$ to 2 hours. The carbon dioxide was collected in standard potash and estimated by titration. The following figures indicate the total amount of carbon dioxide obtained from fresh minced muscle when treated as described.

No.	Animal	Time after death in minutes	No. of gms. taken	No. of c.c. obtained
1	Rabbit	12	100	21·84
2	,,	9	100	22·4
3	,,	10	100	16·24
4	Guinea-pig (with bones)	7	178	97·5

In many other experiments done with rabbit's muscle the average amount of carbon dioxide obtained varied from 16 c.c. to 25 c.c. per 100 gms. of muscle.

In order therefore to determine the true oxidation it was necessary to estimate the total carbon dioxide evolved by boiling with weak acid in a control experiment. At the same time an equivalent weight of minced tissue was placed in the flask of the apparatus and kept in the presence of oxygen for a given time. The carbon dioxide given out and the oxygen absorbed were then estimated, and the tissue subsequently boiled with weak acid in the same way as the control, the amount of carbon dioxide obtained from this being added to that obtained from the tissue incubated in oxygen. The total amount minus the control carbon dioxide gave the carbon dioxide formed by oxidation. This was generally nearly the same as the oxygen absorbed, though certain

variations were found, the respiratory quotient varying from about 0·9 to 1·3. An example of such an experiment is given here.

Exp. 1. 80 gms. minced rabbit's muscle obtained immediately after death were boiled for 1½ hours with weak HCl.

Total CO_2 obtained = 13·38 c.c.

80 gms. of same muscle in oxygen at 38° C. for 3 hours yielded 25 c.c. CO_2 and absorbed 11 c.c. O_2 and on subsequent boiling gave 2·8 c.c. CO_2.

Total yield of CO_2 = 27·8 c.c.

And 27·8 c.c. − 13·38 c.c. (amount originally present) = 14·50 c.c. CO_2 due to oxidation.

$$\therefore\ RQ = \frac{14{\cdot}50}{11} = 1{\cdot}3.$$

Experiments were now conducted to ascertain whether the addition of glucose to the minced tissue had any effect in raising the carbon dioxide output, or increasing the amount of oxygen absorbed. Two equal portions of minced tissue were treated in exactly the same way, the only difference being that a certain amount of glucose was added to one flask. The results, however, were entirely negative, for the tissue to which sugar had been added gave practically the same figures as the control, and in no case did the addition of sugar change the carbon dioxide output or the oxygen intake. Other substances, such as glycogen, sodium lactate, and sodium pyroracemate also gave negative results.

Exp. 2. 80 gms. muscle + 100 c.c. blood + ·5 gm. glucose absorbed 8 c.c. O_2 in 2 hours.
,, ,, ,, no glucose ,, 7·2 ,, ,,

Exp. 3. 80 gms. muscle + 160 c.c. blood + 1 gm. glycogen absorbed 31·4 c.c. O_2 in 3 hours.
,, ,, ,, no glycogen ,, 33·2 ,, ,,

Exp. 4. 45 gms. muscle + 100 c.c. sol. of sodium lactate[1] + 100 c.c. blood absorbed 17·6 c.c. O_2 in 3 hours.

45 gms. muscle + 100 c.c. sol. of sodium pyroracemate[2] + 100 c.c. blood absorbed 10·6 c.c. O_2 in 3 hours.

45 gms. muscle + 100 c.c. blood absorbed 19·5 c.c. O_2 in 3 hours.

It would thus seem that minced tissues when used immediately after death possess no greater power of forming carbon dioxide from added sugar in an atmosphere of oxygen than they have in an atmosphere of carbon dioxide or hydrogen. In this connection it is exceedingly important to use fresh tissue and to limit the duration of the experiment to three hours or less; the difficulties described in our former paper are exaggerated when tissue is kept in an atmosphere of oxygen,

[1] 5·6 gms. Kahlbaum's Syrup to 500 c.c. salt solution.

[2] 4·4 gms. Kahlbaum's Acid neutralised to litmus and made up to 500 c.c. with salt solution.

and as the addition of glucose to a minced organ provides a better nutrient medium for bacteria apparently positive results are sometimes obtained which in reality are due to infection. In every case in which any difference was found in our experiments bacteria were proved to be present; where bacteria were absent sugar produced absolutely no effect.

Experiments with expressed juice of tissues.

With a view to ascertaining something of the nature of the oxidisable substance in the tissues from which the carbon dioxide was produced many experiments were carried out and an endeavour was made to ascertain whether expressed tissue juice was capable of respiration.

Muscle and tissue juices were obtained by mincing up the fresh substance, grinding it in a mortar with silver sand, and kieselguhr, and subjecting this to high pressure in the usual way. This juice was then tested, but in no case was there any evidence of respiratory activity, as seen in the following results.

Exps. 5–9. The minced muscle or organ was thoroughly ground for 10 minutes with an equal weight of sand. This was then mixed with kieselguhr and ground for 10 to 15 mins. more. The powdery mass which resulted was subjected to a pressure of from $2\frac{1}{2}$ to 3 tons per square inch, and the juice obtained incubated at 38° C. in an atmosphere of oxygen.

Exp.	Animal	Substance used	Time incubated in hours	Absorption of O_2 in c.c.
5	Rabbit	90 c.c. juice (muscle)	2	0
	,,	100 c.c. ,, ,, ...	2	1·3
6	Pigeon	58 c.c. ,, ,, +100 c.c. ox-blood	2	4·4
7	Rabbit	70 c.c. ,, ,, ,, ,,	2	1·6
	,,	100 c.c. ,, ,, ...	2	1·2
8	Ox	100 c.c. blood	2	3
9	Rabbit	100 c.c. juice (muscle)	2	2·2

The amount of juice obtained from the same weight of tissue varied considerably, but in general from 200 to 250 gms. rabbit's muscle were required to yield 100 c.c. juice. The oxygen absorption figures are so small as to be insignificant; in cases in which blood was used they are to a certain extent due to this, for blood alone is capable of absorbing a small quantity of oxygen (Exp. 8). On the other hand salt solution alone may absorb from 1 to 2 c.c. of oxygen, so that the results are practically negative.

Since, however, a great deal of mechanical manipulation was necessary in order to disintegrate the tissues previous to the juice

being expressed, some experiments were made to determine whether this interference destroyed or reduced the respiratory activity. It was found that when the minced tissue was rubbed in the mortar without sand no appreciable diminution was in evidence, but when sand was used the resulting mass was less active than the original minced muscle. There was a distinct reduction both in the amount of carbon dioxide given out and in the amount of oxygen absorbed. The following figures were obtained for oxygen absorption.

Exp. 10. 100 gms. minced muscle (rabbit) + 100 gms. sand subsequently added absorbed 17 c.c. O_2 in 3 hours.

100 gms. minced muscle (rabbit) thoroughly *ground* with 100 gms. sand absorbed 13 c.c. O_2 in 3 hours.

Exp. 11. 90 gms. minced muscle (rabbit) + 90 gms. sand absorbed 10 c.c. in 3 hours.

90 gms. minced muscle (rabbit) *ground* with 90 gms. sand absorbed 6 c.c. in 3 hours.

This difference, however, was not very great, and did not explain the inactivity of tissue juice. Experiments carried out with magnesium hydrate added to the sand in order to prevent the usual development of acidity gave no better result. The reduced activity is probably due to direct interference with the integrity of the muscle cell. Though the tissue, when ground up with sand, was still fairly active it was found that the subsequent addition of much kieselguhr had a marked effect in lowering oxidation.

The powdered substance prepared in the usual way with sand and kieselguhr was much less active than the control without kieselguhr; in some cases it seemed to have little or no respiratory activity whatever. Thus:

Exp. 12. 100 gms. minced rabbit's muscle + 100 gms. sand ground for 10 minutes and then mixed with 30 gms. kieselguhr and ground for 5 minutes gave on incubation in oxygen with 100 c.c. blood an absorption of 4·6 c.c. oxygen in 2 hours.

100 gms. rabbit's muscle thoroughly minced + 100 gms. sand + 100 c.c. blood absorbed 14·8 c.c.

Exp. 13. 100 gms. rabbit's muscle minced and ground in mortar for 5 minutes with 10 gms. kieselguhr incubated as above with 100 c.c. blood absorbed 9 c.c. oxygen in 2 hours.

100 gms. minced muscle + 100 c.c. blood absorbed 15·8 c.c. oxygen in 2 hours.

This effect of kieselguhr is difficult to understand, but it suggests that we are here dealing with a substance, possibly of enzymic nature, which is adsorbed or otherwise reduced in activity by the kieselguhr.

Since the inactivity of the expressed juice might be due to the insolubility of the respiratory substance, the residual press cake itself was tested, but here again comparatively little effect was obtained;

even the addition of the expressed juice did not result in any appreciable action. Since, however, kieselguhr interferes so markedly with the respiration processes, an attempt was made to obtain juice with a minimum of this substance. A specimen of this mass was tested before being put into the press, and found to be fairly active, but here again the juice was inactive and the press cake alone was considerably reduced in activity, while the addition of juice to the press cake did not appreciably effect the result. Aqueous and saline extracts obtained by thoroughly mixing the minced tissue and fluid and then straining through a cloth were also tried with negative results. The majority of these experiments were carried out on rabbit's muscle. As this is not of itself very active, juice from the pressed breast muscles of pigeons was obtained, but though the original minced substance was exceedingly active, the juice in this case as before gave negative results.

Exp. 14. Breast muscles from six pigeons taken and thoroughly minced.

(*a*) 40 gms. minced muscles + 100 c.c. blood absorbed 41 c.c. O_2 in 2 hours.

Residue was mixed with sand and kieselguhr and pressed. Yield = 58 c.c. juice.

(*b*) 58 c.c. juice absorbed 3·4 c.c. O_2 in 2 hours.

(*c*) 100 gms. press cake + 100 c.c. blood absorbed 4·8 c.c. in 2 hours.

Exp. 15. 110 gms. pigeons' muscles minced thoroughly with knife: added 250 c.c. water and ground thoroughly in mortar. Then pressed through cloth and obtained 260 c.c. extract. Subsequent pressure in press did not give much more juice.

(*a*) 100 c.c. extract + 100 c.c. blood absorbed 4 c.c. O_2 in 2 hours.

(*b*) 100 c.c. extract + 30 gms. press cake + 100 c.c. blood absorbed 11 c.c. in 2 hours.

(*c*) 100 c.c. saline sol. + 30 gms. press cake + 100 c.c. blood absorbed 10 c.c. in 2 hours.

Battelli and Stern make the statement that tissues which have been kept for some hours after death, and in which the oxidation processes are gradually getting less can be re-activated by the addition of an aqueous extract of some fresh tissue. This extract need not be derived from the same kind of substance as that used in the experiment; for example, liver may be activated either by liver juice or by muscle juice.

To the hypothetical substance which they suppose is present in the extract they gave the name 'pnein'(6). We repeated some of these experiments, but failed to obtain any appreciable evidence of the presence of such a phenomenon as they describe. Those experiments were carried out on the muscles and livers of the rabbit and on the livers of the dog, ox and pig, and all with negative results when bacteria were absent. It is noteworthy that Battelli and Stern do not mention the possible presence of bacteria in any of their experiments; in their paper on what they designate as accessory respiration(7) it

is almost certain that in many cases bacteria must have been present in abundance, the organs having been kept for several hours after death in the incubator at 38° C. or left in the body of the animal. If the so-called 'accessory respiration' is not altogether the result of bacterial infection it is certain from the method adopted that the results obtained must have been vitiated by bacterial action.

Experiments with minced tissues. Since tissue juice failed to show any definite respiratory action a number of experiments were carried out on the minced tissues. In common with other observers, it was found that the activity of the tissue was greatly increased by the addition of blood. As a general rule minced tissue to which blood was added was from two to four times as active as the same tissue when incubated in saline solution. This effect was proved to be dependent on the hæmoglobin present, for isolated hæmoglobin prepared from horse's blood gave the same effect as an equivalent amount of blood. This is shown in the following experiment.

Hæmoglobin was prepared from horse's blood according to the directions given by Schulz[8]. The crystalline substance obtained contained a considerable amount of ammonium sulphate, but since experiment showed that this salt did not appreciably affect the respiration, no attempt was made to remove it. A solution of the hæmoglobin obtained was of such strength that 70 c.c. of it were equal to 28 c.c. of blood, and 1 c.c. contained 0·04 gm. ammonium sulphate.

Exp. 16. 50 gms. minced rabbit muscle + 28 c.c. blood + 2·8 gms. ammonium sulphate + 72 c.c. salt solution absorbed 6·4 c.c. O_2 in 2 hours.

50 gms. minced rabbit muscle + 70 c.c. Hb sol. + 30 c.c. salt sol. absorbed 6·3 c.c. O_2 in 2 hours.

The maximum effect was obtained when blood was used in the proportion of about 100 c.c. to 40 gms. tissue. When only 50 c.c. blood were used the effect was generally somewhat less, but the results were by no means constant. Hæmolysed blood acted in just the same way as ordinary blood. Again, blood from some other animal was quite as efficient as the animal's own blood, and the addition of even a few cubic centimetres to the minced tissue increased the oxidation appreciably. Changes in the reaction of the tissue produced little or no change; any slight differences observed were very irregular and indefinite, and too small to be of any practical importance. Since bacteria were very troublesome, several antiseptic substances, such as toluene, chloroform etc. were tried, but they all depressed the tissue oxidation processes. A typical example is as follows:

Exp. 17. 100 gms. minced muscle + 100 c.c. normal salt solution absorbed 5 c.c. O_2 in 2 hours.

100 gms. minced muscle + 100 c.c. normal salt solution + 2 c.c. toluene absorbed 1·2 c.c. O_2 in 2 hours.

TABLE I.

No.	Animal	Tissue	Amount of tissue in gms.	Time after death	Fluid in which incubated	No. of c.c.s O_2 absorbed in 2 hours	No. of c.c.s of O_2 absorbed per 100 gms. tissue per hour
1 (*a*)	Dog	Muscle	40	15 mins.	100 c.c. blood (ox)	39·8	49·7
1 (*b*)	,,	Liver	40	,,	,, ,,	31·9	39·8
1 (*c*)	,,	Kidney	40	,,	,, ,,	24·6	30·7
1 (*d*)	,,	Muscle	40	3 hrs. in cold room	,, ,,	20·2	25·2
1 (*e*)	,,	Muscle	40	,,	100 c.c. blood (dog)	19·9	24·8
1 (*f*)	,,	Liver	40	,,	100 c.c. blood (ox)	18·4	23
2 (*a*)	Pigeon	Muscle	40	13 mins.	100 c.c. norm. salt sol.	18·6	23·2
2 (*b*)	,,	Muscle	40	,,	100 c.c. blood (ox)	41	51·2
3 (*a*)	,,	Muscle	40	10 mins.	,, ,,	68·3	85·3
3 (*b*)	,,	Liver	16	,,	40 ,, ,,	7·8	24·4
4 (*a*)	Cat	Muscle	40	15 mins.	100 c.c. norm. salt sol.	5·8	7·2
4 (*b*)	,,	Muscle	40	,,	100 c.c. blood (ox)	23·8	29·7
4 (*c*)	,,	Liver	40	,,	,, ,,	16·2	20·2
5 (*a*)	,,	Muscle	40	14 mins.	,, ,,	25	31·2
5 (*b*)	,,	Liver	40	,,	,, ,,	19	23·7
6 (*a*)	Sheep	Muscle(heart)	40	1¼ hrs.	,, ,,	17·4	21·7
6 (*b*)	,,	Liver	40	,,	,, ,,	30	37·5
6 (*c*)	,,	Kidney	40	,,	,, ,,	37·2	46·5
7 (*a*)	,,	Pancreas	40	1½ hrs.	,, ,,	18·6	23·2
7 (*b*)	,,	Liver	40	,,	,, ,,	27·6	34·5.
8 (*a*)	Ox	Liver	40	1 hr.	,, ,,	24·2	30·2
8 (*b*)	,,	Pancreas	40	,,	,, ,,	13·4	16·7
9 (*a*)	,,	Kidney	40	1½ hrs.	,, ,,	22·4	28
9 (*b*)	,,	Liver	40	,,	,, ,,	20	25
9 (*c*)	,,	Pancreas	40	,,	,, ,,	9·6	12
10 (*a*)	Kid	Muscle	40	25 mins.	,, ,,	42·2	52·7
10 (*b*)	,,	Liver	40	,,	,, ,,	38·7	48·3
10 (*c*)	,,	Kidney	24	,,	50 c.c. blood (ox)	32	66·7
11 (*a*)	Calf	Muscle	40	1½ hrs.	,, ,,	15·2	19
11 (*b*)	,,	Liver	40	,,	,, ,,	25·2	31·5
11 (*c*)	,,	Pancreas	40	,,	,, ,,	16·4	20·5
12 (*a*)	Guinea-pig	Muscle	40	10 mins.	,, ,,	24·2	30·2
12 (*b*)	,,	Liver	40	,,	,, ,,	22	27·5
13 (*a*)	Rabbit	Muscle	100	9 mins.	,, ,,	28	14
14 (*a*)	,,	Muscle	40	10 mins.	,, ,,	8	10

In the experiments of Battelli and Stern the figures obtained from minced animal tissues were exceedingly high. Experiments of the same nature carried out by means of our apparatus gave figures very much smaller than those obtained by the above observers.

The results of some experiments from each animal used is given below. The animal was killed and some of the tissues separated and minced as quickly as possible after death. They were then incubated in the ordinary way in an atmosphere of oxygen at 38° C. for two hours. Sometimes parts of the tissues were kept in the cold room for some hours, and then minced and tested: even after this time oxidation was fairly active. Attempts were also made to examine tissues kept at the laboratory temperature for a few hours but bacteria greatly interfered with the results.

The absorptions given were obtained in two hours, and the half of this is taken in calculating results per hour. It might be objected that oxidation during the first hour is much more active, but in general little difference has been observed. After the second hour, however, the tissue gradually loses activity, but later results are generally complicated by bacterial action so that no reliance can be placed upon them.

The figures given in Table I, agree in a general way with those obtained by Battelli and Stern; thus pigeon muscle gives much higher values than rabbit's muscle, while sheep and ox are intermediate. On the other hand, the absolute values for each tissue are very different, and never amount even approximately to the values given by these observers. The general conclusion from these figures is that the post mortem oxidation processes are not sufficiently active to render it easy to detect the oxidisable substance; a very small amount of the latter would be sufficient to account for all the oxidation found.

Summary

1. An apparatus for estimating the post mortem gaseous metabolism of minced organs is described in the paper.

2. Minced organs and tissues of animals have no more power of producing carbon dioxide from sugar when incubated in an atmosphere of oxygen than they have in an atmosphere of nitrogen or hydrogen.

3. Tissue juices prepared by the aid of kieselguhr and aqueous and saline extracts of tissues possess little or no respiratory activity.

4. The oxidation processes of minced tissue are materially lowered by grinding up the tissue with sand or by mixing it with kieselguhr; antiseptics act in the same direction.

5. The figures obtained by us for the oxygen absorption and carbon dioxide output of minced tissues are considerably lower than those obtained by Battelli and Stern.

REFERENCES.

(1) Harden and MacLean. This Journal, XLII. p. 64. 1911.
(2) Battelli and Stern. Journ. de Physiol. et de Path. gén. IX. p. 34. 1907.
(3) Thompson. Private communication.
(4) Pflüger. Pflüger's Arch. XVIII. pp. 381, 247. 1878.
(5) Stintzing. Pflüger's Arch. XVIII. p. 388. 1878.
(6) Battelli and Stern. Soc. Biol. LXV. p. 489. 1908.
(7) Battelli and Stern. Biochem. Ztsch. XXII. p. 486. 1910.
(8) Schulz. Ztsch. f. physiol. Chem. XXIV. p. 455. 1898.

[*Reprinted from the* PROCEEDINGS OF THE ROYAL SOCIETY, B. Vol. 84]

The Bacterial Production of Acetylmethylcarbinol and 2.3-Butylene Glycol from Various Substances.

By ARTHUR HARDEN, F.R.S., and DOROTHY NORRIS.

(Received November 22, 1911,—Read February 1, 1912.)

(From the Biochemical Department, Lister Institute.)

In working out the action of *B. lactis aërogenes* on glucose quantitatively, Harden and Walpole (1) found that, in addition to the products already noted in the action of *B. coli communis* on glucose (2), a small quantity of acetylmethylcarbinol, $CH_3{\cdot}CH(OH){\cdot}CO{\cdot}CH_3$, and a considerable proportion of 2.3-butylene glycol, $CH_3{\cdot}CH(OH){\cdot}CH(OH){\cdot}CH_3$, were formed, the latter corresponding to about 33 per cent. of the carbon of the sugar fermented. The production of acetylmethylcarbinol by the action of *Tyrothrix tennis*, *B. subtilis* and *B. mesentericus vulgatus*, and similar organisms on glucose, had been previously noted by Grimbert (3) and by Desmots (4).

The presence of acetylmethylcarbinol is of especial interest, as it has been shown to be the substance responsible for the Voges and Proskauer reaction (5). In view of this fact, and of the interest attaching to this mode of decomposition of glucose, it becomes a matter of some importance to discover what substances will give rise to acetylmethylcarbinol and butylene glycol during fermentation, and also which bacteria are capable of producing these two compounds. *B. lactis aërogenes* and *B. cloacæ* have been shown to

produce both substances from glucose, mannitol, and fructose (6, 7, 8); hence, in the first place, the action of these organisms was studied, and similar experiments were later carried out with *B. coli communis*, but with negative results.

It was of especial interest to discover whether these substances could be produced from carbon compounds less complex than the hexoses, and a variety of simpler substances containing fewer carbon atoms were therefore tried, *e.g.*, glycerol, ethylene glycol, malic acid, etc.

The formation of acetylmethylcarbinol or butylene glycol from substances containing three or fewer carbon atoms would necessarily involve a carbon synthesis which would be of considerable theoretical interest. An instance of this kind, the production of butyric acid and butyl alcohol from lactic acid and from glycerol in the butyric fermentation, has long been known (9, 10). To explain this, it has been supposed that acetaldehyde is first formed and then serves as the source of the butyl alcohol and butyric acid. The aldehyde may be supposed to undergo an aldol condensation followed by reduction, with or without subsequent oxidation:

$$2CH_3{\cdot}CHO = CH_3{\cdot}CH(OH){\cdot}CH_2{\cdot}CHO.$$

$$CH_3{\cdot}CH(OH){\cdot}CH_2{\cdot}CHO + 4H = CH_3{\cdot}CH_2{\cdot}CH_2{\cdot}CH_2{\cdot}OH + H_2O.$$

It seems possible that the production of acetylmethylcarbinol and butylene glycol may be due to a somewhat similar course of events, which may be represented as follows, although these reactions have not, hitherto, been observed in the laboratory :—

$$2CH_3{\cdot}CHO + 2H = CH_3{\cdot}CH(OH){\cdot}CH(OH){\cdot}CH_3.$$

$$2CH_3{\cdot}CHO = CH_3{\cdot}CO{\cdot}CH(OH){\cdot}CH_3.$$

Experiments to test this hypothesis were made, with the result that the production of the glycol by bacterial action from acetaldehyde was conclusively established, although the mechanism of this production has not yet been ascertained.

It by no means necessarily follows, however, that in the fermentation of glucose the butylene glycol is actually produced from preformed acetaldehyde.

1. *Experimental Methods.*

As a general rule the culture medium consisted of 1 per cent. Witte's peptone water containing 5 per cent. of the substance under investigation. Sufficient chalk was added to neutralise any acid formed during fermentation, and after inoculation the culture was grown for three weeks at 37° C. under anaërobic conditions, the flask being fitted with a mercury trap to allow the escape of any gases.

Among the products of the reaction, acetylmethylcarbinol and butylene glycol only have been estimated quantitatively. A more complete investigation has, however, been made in the case of glycerol, and the results obtained will form the subject of another communication.

To ascertain whether the carbinol and glycol were derived from the acetaldehyde, sugar or other substances in question, and not simply from a constituent of the peptone, control experiments were carried out with peptone water alone as culture medium. In no case could the slightest trace of either substance be detected.

2. *Detection and Estimation of Acetylmethylcarbinol and Butylene Glycol.*

As some of the substances employed could only be used in small quantities, it was necessary to elaborate a method for the detection of both acetylmethylcarbinol and butylene glycol in small amounts. Attempts were first made to separate these two compounds by cautious evaporation and estimate them individually, but it was found impossible to arrive at any quantitative values in this way.

The method ultimately adopted was as follows :—

The medium in which the organism had been grown was carefully distilled to as small a bulk as possible over a free flame and then to dryness under reduced pressure at 37° C. The distillates were then united and made up to a definite volume and portions tested for reducing power and for Voges and Proskauer's reaction, which, as stated above, is due to the presence of acetylmethylcarbinol. To perform this reaction 3 c.c. of 1 per cent. Witte's peptone water are mixed in a test-tube with an equal quantity of 10 per cent. caustic soda, 2 c.c. of the solution to be tested are then carefully poured on to the surface of the liquid, and the tube is allowed to stand at room temperature. If acetylmethylcarbinol be present, a pink ring forms at the juncture of the two liquids. With very small quantities of carbinol, this may take some hours to develop, but with larger amounts the colour soon appears and quickly spreads through the whole of the solution, a green fluorescence being also produced. It was found that more delicate results could be obtained by the above method than by simply mixing the solutions.

Experiments in connection with other work, shortly to be published, have indicated that the carbinol is alone responsible for the reducing power of the distillates, and hence an estimation of this by Bang's (11) method at once gives the amount of acetylmethylcarbinol present, the reducing power of this substance being known (12).

The estimation of the butylene glycol is not such a simple matter.

A small amount is held in the dry residue after distillation, and must be extracted with dry ether, and the glycol thus obtained, after evaporation of the ether, added to the distillate.

The estimation depends on the fact that the glycol is readily oxidised by bromine under the influence of light to diacetyl, which can be estimated by its reducing power. Any acetylmethylcarbinol present is quantitatively oxidised to diacetyl in this way, but as the quantity of this substance will already have been determined, the amount of diacetyl formed from it is known, and the difference between this and the total diacetyl represents that due to the glycol. The details of the process are as follows:—An aliquot portion of the distillate is treated with a small quantity of bromine (0·1 c.c. for the distillate from the fermentation of 5 grm. of substance), and left exposed for 12 to 15 hours to the light of a 50-candle-power electric lamp. A further addition of bromine is made if the solution becomes completely decolorised, and the exposure continued. Any free bromine is then removed by the cautious addition of sulphurous acid, excess of this being carefully avoided. The solution is next saturated with sodium chloride, and the diacetyl distilled off and estimated by determining the reducing power of the distillate.

The results obtained by the above method are low, as the oxidation of the glycol to diacetyl is not quantitative. Control experiments with known amounts of glycol show that the actual results obtained are two-thirds the correct value, and a correction for this has been made in the tables given below.

In most cases the diacetyl produced by the oxidation was further characterised by the preparation and analysis of the phenylosazone.

Two typical analyses gave the following results:—

1. Substance fermented—galactose. Organism—*B. cloacæ*. Osazone—m.p. 243° C.

0·2109 grm. gave 39·4 c.c. N at 23·5° C. and 765 mm. N = 21·14 per cent.

2. Substance fermented—arabinose. Organism—*B. lactis aërogenes*. Osazone—m.p. 244° C.

0·1349 grm. gave 25 c.c. N at 22° C. and 765 mm. N = 21·13 per cent.

Diacetylphenylosazone, $C_{16}H_{18}N_4$, requires N = 21·05 per cent.

In the sugar experiments the amount unfermented was estimated in the residue left after the extraction of the glycol by dissolving it in water, making up to a known volume, and determining the reducing power after treatment with mercuric nitrate (Patein).

3. *Results Obtained.*

The results obtained with the organisms employed are indicated in the tables given below. The figures given in Columns 8 and 9 are the values calculated from the actual results found for 10 grm. of sugar fermented.

As previously mentioned, the results obtained with *B. coli communis* were in every case negative.

Table I.—Action of *B. lactis aërogenes* (Escherich) and *B. cloacæ* (Jordan) on various Sugars.

Experiment.	Organism.	Sugar.	Weight fermented.	Time of growth.	Carbinol per 10 grm. sugar fermented.	Glycol per 10 grm. sugar fermented.
			grm.		grm.	grm.
1	*B. lactis aërogenes*	Glucose	4·66	3 weeks	0·11	1·42
2	,, ,,	,,	4·85	,,	0·12	1·39
3	,, ,,	,,	5·00	,,	0·11	1·36
4	,, ,,	,,	2·47	,,	0·11	1·44
5	*B. cloacæ*	Fructose	4·67	,,	0·11	1·55
6	,,	,,	4·15	,,	0·11	1·51
7	,,	Mannose	2·32	,,	0·11	1·36
8	*B. lactis aërogenes*	,,	2·45	,,	0·10	1·42
9	,, ,,	Galactose	4·03	,,	0·06	0·86
10	,, ,,	,,	2·40	,,	0·07	0·85
11	*B. cloacæ*	,,	4·74	,,	0·08	1·41
12	,,	,,	4·38	,,	0·08	1·36
13	*B. lactis aërogenes*	Arabinose	3·92	,,	0·07	1·18
14	,, ,,	,,	2·39	,,	0·08	1·19
15	*B. cloacæ*	,,	5·00	,,	0·06	1·19
16	,,	,,	1·83	,,	0·08	1·18
17	*B. lactis aërogenes*	Isodulcite	2·03	32 days	0·43	1·45
18	,, ,,	,,	1·77	3 weeks	0·67	1·54
19	*B. cloacæ*	,,	2·03	,,	0·04	0·81

It will be seen from the above table that glucose, fructose, and mannose have given practically the same quantities of carbinol and glycol respectively, whilst with galactose the carbinol is slightly less in amount with both organisms used, and the yield of glycol varies with the organism employed. The results obtained in the case of arabinose show close agreement for both organisms, although in all four cases lower figures are obtained than with the hexoses. The question of isodulcite is interesting, as the amount of carbinol obtained by means of *B. lactis aërogenes* is abnormally high compared with that obtained from the other sugars or by using *B. cloacæ*.

The galactose and arabinose used in the above experiments were previously purified from traces of glucose by fermentation with yeast.

The amount of glycol produced from glucose is decidedly lower than that obtained by Harden and Walpole, a result which is possibly due to the employment of a different strain of the organism.

Table II.—Action of *B. lactis aërogenes* (Escherich) and *B. cloacæ* (Jordan) on various Alcohols.

Experiment.	Organism.	Alcohol.	Weight taken.	Carbinol per 10 grm. taken.	Glycol per 10 grm. taken.
			grm.	grm.	grm.
20	*B. lactis aërogenes*	Glycerol	5	Nil	0·05
21	„ „	„	50	„	0·05
22	„ „	„	10	„	0·05
23	„ „	„	10	„	0·04
24	*B. cloacæ*	„	10	„	Nil
25	*B. lactis aërogenes*	Ethylene glycol	5	„	0·08
26	„ „	„	5	„	0·08
27	„ „	„	5	„	0·09
28	„ „	Adonitol	2·50	0·06	0·22
29	„ „	„	2·50	0·06	0·24
30	„ „	Mannitol	5	0·03	0·23
31	„ „	„	5	0·03	0·04

The growth was continued for three weeks, except in the case of Experiment 31, which was for two weeks. The residual alcohols were not estimated, so that the results stated above are not corrected for amount of substance unfermented. Only in the case of mannitol and adonitol was any carbinol detected. All these alcohols, however, yielded glycol. Citric and malic acids gave negative results with both organisms.

The action of *B. lactis aërogenes* on dihydroxyacetone, $CH_2(OH)\cdot CO\cdot CH_2OH$, was also tried, but here again without positive result.

4. *Experiments on the Synthesis of* 2.3-*Butylene Glycol from Acetaldehyde by means of* B. lactis aërogenes.

For each experiment a litre of Witte's peptone water was made up and sufficient chalk added to neutralise any acids which might be formed during fermentation. The medium was then inoculated with *B. lactis aërogenes* and incubated at 37° C. for 24 hours before any addition of acetaldehyde was made, in order to establish a good growth of the organism.

In some experiments calcium formate (10 grm. formic acid per 100 c.c. water neutralised with $CaCO_3$) was added with the acetaldehyde (10 grm. per 100 c.c.); in others acetaldehyde was used alone.

Two cubic centimetres of the above solutions were added to the culture medium each day under sterile conditions, so that each flask received a daily addition of 0·2 grm. of acetaldehyde and the same quantity of formic acid (as formate) in the cases where this was used.

This treatment was continued until in two experiments 60 c.c. of the above solutions had been added, and in two others until 80 c.c. had been

added. At the end of this time and occasionally during the progress of the experiment sub-cultures were made to show that the organism in question was still alive and uncontaminated. In two experiments the whole of the acetaldehyde had not been used up, so that the liquid obtained by distillation of the culture medium was strongly reducing to Fehling's solution. In two other cases sufficient time was allowed to elapse between the last addition of aldehyde and the examination of the products to ensure the complete removal of this factor.

In these two cases the distillates obtained had no reducing power. In every experiment the distillate was tested by means of the Voges and Proskauer reaction for acetylmethylcarbinol, and in no case could any trace of this be detected.

The liquid was further examined for butylene glycol, which was in every case found to be present, though in extremely small quantities. It was detected as described above by the formation of diacetyl, this substance being proved to be present in every case by the positive results given by the Voges and Proskauer reaction after oxidation of the culture distillate.

In one case sufficient osazone was prepared from the diacetyl for a determination of the melting point, which was found to be 244° C. Among the products of the reaction were also found ethyl alcohol, acetic acid, and some succinic acid. Lactic acid was not present. These products were estimated in the manner previously described by Harden (13) with a few slight modifications. In the estimation of the alcohol any unchanged acetaldehyde present was removed by oxidation with moist silver oxide. The acetic acid was determined by the method of Macnair (14).

The table below shows the results obtained in three typical experiments:—

Table III.

	1.	2.	3.
Total acetaldehyde added	6·07 grm.	8·0 grm.	7·6 grm.
Total formic acid added (as Ca formate)	6·07 „	Nil	7·6 „
	grm.	grm.	grm.
Alcohol	6·5	4·02	4·5
Acetic acid	2·5	1·38	1·3
Succinic acid	Not estimated	1·25	0·12
Butylene glycol	+	0·677	0·109

From the point of view of the production of butylene glycol the presence of calcium formate appears to be detrimental, and it is also interesting to note the somewhat large quantity of succinic acid produced in Experiment 2, which also gave the largest yield of glycol.

Summary of Results.

1. *B. lactis aërogenes* and *B. cloacæ*, when grown in a peptone solution containing either glucose, fructose, mannose, galactose, arabinose, isodulcite, mannitol or adonitol, produce both acetylmethylcarbinol and 2.3-butylene glycol.

2. Glycerol, ethylene glycol and acetaldehyde, under similar conditions, also give rise to 2.3-butylene glycol in presence of *B. lactis aërogenes*, but no acetylmethylcarbinol is produced. In these three cases a carbon synthesis is involved, analogous to that which occurs in the butyric fermentation of glycerol and lactic acid.

3. The fermentation of citric and malic acids, of dihydroxyacetone, and of peptone water, gives rise to neither carbinol nor glycol.

REFERENCES.

1. Harden and Walpole, 'Roy. Soc. Proc.,' 1906, B, vol. 77, p. 399.
2. Harden, 'Chem. Soc. Journ.,' 1901, p. 610.
3. Grimbert, 'Compt. Rend.,' 1901, vol. 132, p. 706.
4. Desmots, 'Compt. Rend.,' 1904, vol. 138, p. 581.
5. Harden, 'Roy. Soc. Proc.,' 1906, B, vol. 77.
6. Harden and Walpole, 'Roy. Soc. Proc.,' 1906, B, vol. 77, p. 399.
7. Walpole, 'Roy. Soc. Proc.,' 1911, B, vol. 83, p. 272.
8. Thompson, 'Roy. Soc. Proc.,' this vol., p. 500.
9. Fitz, 'Ber.,' 1880, vol. 13, p. 1309.
10. Buchner and Meisenheimer, 'Ber.,' 1908, vol. 41, p. 1410.
11. Bang, 'Biochem. Zeitschr.,' 1907, vol. 2, p. 271.
12. Harden and Walpole, 'Roy. Soc. Proc.,' 1906, B, vol. 77, p. 399.
13. Harden, 'Chem. Soc. Journ.,' 1901, p. 610.
14. Macnair, 'Chem. News,' 1887, vol. 55, p. 229.

Harrison and Sons, Printers in Ordinary to His Majesty, St. Martin's Lane.

[*Reprinted from the* PROCEEDINGS OF THE ROYAL SOCIETY, B. Vol. 85]

The Bacterial Production of Acetylmethylcarbinol and 2.3-Butylene Glycol from Various Substances.—II.*

By ARTHUR HARDEN, F.R.S., and DOROTHY NORRIS, Biochemical Department, Lister Institute.

(Received January 25,—Read February 29, 1912.)

The action of *B. subtilis* (Cohn), *B. mesentericus vulgatus* (Flügge) (*B. vulgatus* (Flügge) Migula), and *Tyrothrix tenuis* (Duclaux) (*B. tenuis* (Duclaux) L. and N.) on various substances has been investigated by Péré (1). This observer, on distilling his various culture media, obtained lævo-rotatory distillates strongly reducing to Fehling's solution. In all cases he concluded that the volatile substance present was glyceraldehyde, and upon his results based a theory that sugars undergoing bacterial fermentation break down primarily to a triose, that is to say, glycerose.

He was, however, unable to characterise his compound satisfactorily; for example, it did not give Schiff's reaction, no osazone was obtainable, and although in some cases he obtained small quantities of lead, calcium, and barium salts of an acid formed by the oxidation of his volatile substance with nitric acid, which he took for salts of glyceric acid, the quantities analysed were so small that no reliance can be placed upon the results. Moreover none of his salts was obtained in crystalline form.

Soon after the appearance of Péré's work Wohl (2) succeeded in preparing glyceraldehyde in a pure state, and found that it was non-volatile in steam, that it gave Schiff's reaction, and also formed a highly characteristic osazone, M.P. 131° C. It seemed therefore impossible that the volatile substance obtained by Péré could have been glyceraldehyde, and a further investigation of the subject has therefore been made.

In a previous communication (3) the action of *B. lactis aërogenes* and *B. cloacæ* on many carbohydrates, alcohols, etc., has been described. With glycerol itself the liquid obtained on distilling the culture medium was absolutely without reducing power. In the majority of cases, however, the distillate possessed reducing power which was shown to be due not to the presence of glyceraldehyde, but to that of acetylmethylcarbinol, $CH_3{\cdot}CH(OH){\cdot}CO{\cdot}CH_3$, the substance responsible for the Voges and Proskauer reaction (4), which had previously been observed as a product of the bacterial fermentation of glucose by Grimbert (5). The above experiments were carried out under anaërobic conditions, but Walpole (6) has shown that

* For first part see paper read February 1, B, vol. 84. p. 492.

the yield of carbinol may be increased by aërobic culture. The whole of Péré's experiments were carried out under aërobic conditions, and although he employed different organisms from those given above, they have all been shown by Desmots (7) to be capable of producing acetylmethylcarbinol under suitable circumstances. Desmots, in fact, describes experiments very similar to those carried out by Péré, but makes no mention of the possible formation of glyceraldehyde. It seems therefore not at all unlikely that Péré's volatile reducing substance was acetylmethylcarbinol and not glyceraldehyde. His experiments have accordingly been repeated, using identical culture media and conditions of growth, and the results of these investigations form the subject of the present communication. In addition to repeating Péré's experiments a quantitative estimation of the action of *B. lactis aërogenes* on glycerol has been made. In this case neither acetylmethylcarbinol nor glyceraldehyde is obtained.

Action of B. subtilis *and* B. mesentericus vulgatus *on Mannitol and* Tyrothrix tenuis *on Glucose.*

The culture media were made up exactly in the same way as those used by Péré, and in the case of the first two organisms named consisted of 20 grm of mannitol in 200 c.c. water containing 2 grm. ammonium phosphate, 1 grm. ammonium sulphate, and 0·4 grm. potassium phosphate. For the experiment with *Tyrothrix tenuis* 5 grm. of glucose were made up to 100 c.c. with broth. After sterilisation and inoculation with the organism in question, the various culture media were incubated at 37° C. In every case growth was continued for 30 days, after which time the cultures were worked up according to Péré's directions. To take one example—the action of *B. subtilis* on mannitol—after the 30 days' incubation the culture medium was made acid with citric acid and distilled, the distillate was found to be strongly reducing and lævo-rotatory, and also gave a very strong Voges and Proskauer reaction, which is characteristic of acetylmethylcarbinol, but is not given by glyceraldehyde. The remaining distillate was then made alkaline and again distilled, yielding a second time a reducing lævo-rotatory body giving the Voges and Proskauer reaction. This second distillate was then steam-distilled for three hours, and by the end of that time the whole of the reducing body had passed over with the steam, the residue being non-reducing and optically inactive. The steam distillate, on the other hand, was still lævo-rotatory, reducing, and gave the Voges and Proskauer reaction. An osazone was prepared from this distillate and gave a definite melting point of 243° C., corresponding to the phenylosazone of diacetyl, which is always obtained from acetylmethylcarbinol in this manner. In a similar way the action of

B. mesentericus vulgatus and *Tyrothrix tenuis* on mannitol and glucose respectively was also examined and similar results obtained.

The Action of Tyrothrix tenuis *on Glycerol.*

This offered a case of much greater interest, as the formation of acetylmethylcarbinol would involve a carbon synthesis, and does not take place from this substance when *B. lactis aërogenes* is used under anaërobic conditions.

Péré's directions were again carefully followed, 5 grm. of glycerol were made up to 100° C. with broth, and, after sterilisation and subsequent inoculation, were incubated at 37° C. for 30 days. An investigation of the culture medium, as described above, again showed the presence of acetylmethylcarbinol, which passed over into the distillate, and was characterised by the preparation and analysis of the osazone, as well as by the Voges and Proskauer reaction. The lead, calcium, and barium salts of the oxidation products analysed by Péré, and believed by him to be the salts of glyceric acid, were in all probability the salts of lactic acid. As he was only able to analyse extremely small quantities (0·032 grm. of a lead salt containing 50 per cent. Pb) of non-crystalline substances, the experimental error was probably too great for him to distinguish between these two acids. There is therefore no evidence to show that glyceraldehyde is produced in the above fermentations, and the theory that sugars undergoing bacterial fermentation are first broken down to trioses derives no support from this investigation.

The Action of B. lactis aërogenes (*Escherich*) *on Glycerol under Anaërobic Conditions.*

The experiments previously described were all carried out under aërobic conditions, and, as an example of anaërobic decomposition, the action of *B. lactis aërogenes* on glycerol has been studied. This organism under these conditions forms acetylmethylcarbinol from all the hexoses, but produces none from glycerol, no reducing substance at all being found among the products of the reaction.

The method of investigation was substantially that used by Harden (8), the gases, however, being collected and measured in the apparatus devised by Harden, Thompson, and Young (9). The organism was grown in an atmosphere of nitrogen, the medium consisting of 1 per cent. Witte's peptone solution containing 10 per cent. of glycerol, sufficient chalk being added to neutralise the acids formed during fermentation. In the glycerol experiments quoted, the times of growth were respectively five weeks,

two weeks, and four weeks. A different strain of the organism was used in Experiment 1 from that used in Experiments 2 and 3.

The glycerol added, and the amount unfermented, were estimated by means of the method of Zeisel and Fanto (10), as described below.

Estimation of the Residual Glycerol.—100 c.c. of the liquid in which the organism had grown were evaporated at a low temperature under reduced pressure, in order to ensure the removal of all alcohol. The residue was then taken up in a small quantity of water, the volume made up to 100 c.c. with water, and 5 c.c. of this solution taken for the estimation.

Search for Acetylmethylcarbinol.—A portion of the culture medium was distilled and the distillate tested for reducing power with Fehling's solution. In every case the distillate was found to be non-reducing and the absence of acetylmethylcarbinol was further confirmed by trying the Voges and Proskauer reaction, which was invariably negative. It was also found impossible to prepare any osazone.

Detection of 2.3-Butylene Glycol.—This substance was detected and estimated as previously described, the estimation being made on 300 c.c. of the culture medium in which the organism had been grown. In every experiment this glycol was found to be present, the other products being ethyl alcohol, formic, lactic, and succinic acids, carbon dioxide, and hydrogen.

The diacetylphenylosazone prepared from the oxidation product of the butylene glycol was analysed and gave the following results:—

0·1372 grm. substance gave 0·3656 grm. CO_2 and 0·0876 grm. H_2O. C = 72·6 per cent.; H = 7 per cent.

0·0950 grm. substance gave 17·5 c.c. N at 21° C and 765·5 mm. N = 21·12 per cent.

$C_{16}H_{18}N_4$ requires C = 72·2 per cent., H = 6·8 per cent., N = 21·05 per cent.

The lactic acid produced was also characterised by the preparation and analysis of the zinc salt and by Fletcher and Hopkins' reaction (11).

0·1500 grm. of the zinc salt dried at 105° C. gave 0·0499 grm. ZnO. ZnO = 33·28 per cent.

$(C_3H_5O_3)_2Zn$ requires ZnO = 33·4 per cent.

The analysis of the calcium salt prepared from the succinic acid formed gave the following results:—

0·0550 grm. substance gave 0·0202 grm. CaO. CaO = 36·7 per cent.

$(C_4H_4O_4)Ca$ requires CaO = 35·9 per cent. The pure acid was also isolated in this case and gave M.P. 183–4° C.

The percentage of these substances on the weight of glycerol fermented is shown in the following table, Columns 1, 2, and 3. Columns 4 and 5

show for comparative purposes the result of the action of *B. lactis aërogenes* on glucose and mannitol respectively.

Table I.

	Glycerol.			Glucose.	Mannitol.
	1.	2.	3.	4.	5.
Alcohol	35·2	37·7	37·3	17·1	32·5
Acetic acid	6·1	5·0	7·3	5·1	2·5
Lactic acid	13·0	12·7	11·13	5·5	8·6
Succinic acid	4·05	1·6	4·03	2·4	3·2
2.3-Butylene glycol	9·9	Not estimated		(27·2)	(12·0)
Formic acid	6·38	4·9	7·5	1·0	1·5
Carbon dioxide	22·4	28·37	31·8	38·0	35·5
CO_2, c.c. per grm.	110·6	144·0	160·7	198·3	180·3
H_2, c.c. per grm.	79·8	139·0	156·7	82·4	138·3
Ratio H_2/CO_2	0·72	0·97	0·97	0·42	0·77

The figures in brackets are estimated from other experiments.

Table II shows the number of carbon atoms per molecule of glycerol decomposed, represented by each product.

Table II.

	Glycerol.			Glucose.	Mannitol.
	1.	2.	3.	4.	5.
Alcohol	1·42	1·43	1·49	1·34	2·57
Acetic acid	0·18	0·15	0·22	0·31	0·15
Lactic acid	0·40	0·39	0·34	0·33	0·52
Succinic acid	0·12	0·05	0·12	0·15	0·20
2.3-Butylene glycol	0·39	Not estimated		(2·17)	(0·97)
Formic acid and CO_2	0·58	0·69	0·82	1·64	1·53
Total	3·09	2·71	2·99	5·94	5·94
Hydrogen, atoms per molecule	0·65	1·13	1·28	1·33	2·26

Columns 4 and 5 are again comparative ones of *B. lactis aërogenes* on glucose and mannitol.

It is interesting to find that alcohol accounts for 35·2 per cent. of the glycerol used, as against 17·1 per cent. in the case of glucose. Harden suggested (8) that the source of the alcohol might be the presence in the molecule undergoing decomposition of the terminal group $CH_2(OH)\cdot CH(OH)$–. This was confirmed in the case of glucose and mannitol, this latter substance yielding twice the amount of alcohol produced under similar conditions from

glucose. It would be interesting to find whether the same relationship holds in the case of glycerol and glyceraldehyde. Formic acid (or its decomposition products, CO_2 and H_2) and alcohol, which might be formed according to the equation

$$C_3H_8O_3 = C_2H_6O + CH_2O_2,$$

make up 64—77 per cent. of the glycerol fermented.

Summary.

1. The volatile reducing substance obtained by Péré in the aërobic fermentation of mannitol by *B. subtilis* and *B. mesentericus vulgatus*, and of glucose and glycerol by *Tyrothrix tenuis*, is acetylmethylcarbinol, which is readily volatile in steam, gives the Voges and Proskauer reaction, and forms the phenylosazone of diacetyl.

2. The action of *B. lactis aërogenes* on glycerol, under anaërobic conditions, does not give rise to any reducing substance.

The products of this decomposition have been quantitatively estimated and are as follows:—Ethyl alcohol, formic, acetic, lactic and succinic acids, carbon dioxide, hydrogen and 2.3-butylene glycol.

[*Note added February* 29, 1912.—Since writing the foregoing paper our attention has been called to a paper by Fernbach* in which he shows that *T. tenuis* acts both on glucose and glycerol with the production of non-volatile dihydroxyacetone. Volatile reducing substances were also formed which he regards as a mixture of methylglyoxal and formaldehyde. Since neither of these substances is optically active they cannot be identical with the lævo-rotatory substance obtained by Péré and ourselves, so that Fernbach's observations in no way disprove our conclusion that the optically active, volatile substance produced is acetylmethylcarbinol.]

REFERENCES.

(1) Péré, 'Ann. Inst. Past.,' 1896, vol. 10, p. 417.
(2) Wohl, 'Ber.,' 1898, vol. 31, p. 1800.
(3) Harden and Norris, 'Roy. Soc. Proc.,' 1912, B, vol. 84, p. 492.
(4) Harden, 'Roy. Soc. Proc.,' 1906, B, vol. 77, p. 424.
(5) Grimbert, 'Compt. Rend.,' 1901, vol. 132, p. 706.
(6) Walpole, 'Roy. Soc. Proc.,' 1911, B, vol. 83, p. 272.
(7) Desmots, 'Compt. Rend.,' 1904, vol. 138, p. 581.
(8) Harden, 'Chem. Soc. Journ.,' 1901, p. 610.
(9) Harden, Thompson, and Young, 'Biochem. Journ.,' 1910, vol. 5, p. 230.
(10) Zeisel and Fanto, 'Z. anal. Chem.,' 1903, vol. 42, p. 551.
(11) Fletcher and Hopkins, 'Journ. Phys.,' 1907, vol. 35, p. 247.

* 'Compt. Rend.,' 1910, vol. 151, p. 1004.

Harrison and Sons, Printers in Ordinary to His Majesty, St. Martin's Lane.

[*Reprinted from the* Proceedings of the Royal Society, B. Vol. 84]

Action of Dissolved Substances upon the Autofermentation of Yeast.

By Arthur Harden, F.R.S., and Sydney G. Paine.

(Received October 17,—Read December 7, 1911.)

(From the Biochemical Department, Lister Institute.)

During experiments upon the permeability of the yeast-cell it was found that, when yeast was immersed in a molar solution of sodium chloride, and allowed to stand at air temperature, the amount of gas produced by autofermentation was considerably greater than that given by a water control.

The production of carbon dioxide by autofermentation of yeast is brought about by the action of at least two enzymes. The reserve material of the cell, for the most part glycogen, is first converted by a glycogenase into a sugar, which in turn is fermented by zymase with the production of alcohol and carbon dioxide. As the rate of autofermentation is considerably less than that produced by the same yeast in presence of excess of sugar, it follows that the rate of autofermentation is controlled by the rate of production of sugar within the cell, in other words, by the rate of action of the glycogenase. An increase in the rate of autofermentation, therefore, indicates greater activity of this enzyme within the cell. In order to investigate the action of solutions of various salts upon the rate of autofermentation of yeast, this was ascertained by measuring the volume of carbon dioxide evolved during successive intervals of time by means of the apparatus described by Harden, Thompson, and Young (1). The yeast employed was prepared from top-yeast as obtained from the brewery by pressing out the wort in a small hand press, it having been demonstrated (2) that practically the whole of the interstitial liquid can be removed in this way. A certain weight of such pressed yeast was carefully weighed into each of the fermentation flasks, and treated with a certain volume of the various liquids under experiment, controls being made with water. The liquids were saturated with carbon dioxide at 25°, the temperature of the water-bath.

1. *Effect of Sodium Chloride and other Salts upon the Autofermentation of Yeast.*

When yeast was immersed in molar sodium chloride solution the rates of evolution of gas during the first six successive intervals of 20 minutes

were 10·6, 8·4, 6·6, 4·9, 4·8, 4·8 c.c., as against 4·9, 4·2, 3·3, 2·9, 2·8, 2·7 c.c. when the same weight of yeast was immersed in water. In the former case fermentation practically came to an end after six hours, at which time 60 c.c. of gas had been collected as against 31 c.c. from the water control (Table I). In the latter case evolution of gas continued steadily until, after about 60 hours, the volume of gas was identical with that from the sodium chloride experiment.

Table I.—Effect of Sodium Chloride upon the Autofermentation of Yeast.

Time, in hours.	Cubic centimetres of carbon dioxide evolved by 3 grm. of yeast and 20 c.c. of solution.	
	Sodium chloride, molar.	Water control.
1	25·6	12·4
2	40·1	20·8
3	49·6	24·6
4	55·7	27·4
5	58·8	29·4
6	59·7	31·2
24	65·0	49·5
48	67·0	61·5
64	67·5	67·5

This experiment shows that, under the influence of molar sodium chloride, the whole of the fermentable material was decomposed in one-tenth of the time required by the water control.

Experiments were next made in order to determine the optimum concentration of this substance, which would give a maximum rate of autofermentation at the temperature employed.

Table II.—Effect of Varying Concentrations of Sodium Chloride.

No.	Cubic centimetres of carbon dioxide evolved during the first hour from 4 grm. of yeast + 10 c.c. solution											
	Water.	NaCl. 0·5 M.	NaCl. 0·6 M.	NaCl. 0·7 M.	NaCl. 0·8 M.	NaCl. 0·9 M.	NaCl. 1·0 M.	NaCl. 1·1 M.	NaCl. 1·2 M.	NaCl. 1·5 M.	NaCl. 1·7 M.	NaCl. 2 M.
42	21·7	—	—	—	—	—	82·0	—	68·5	57·7	42·5	42·5
47	—	35·6	37·4	41·7	43·1	44·1	42·9	—	—	—	—	—
48	—	—	—	49·0	50·6	50·1	44·7	44·2	41·9	—	—	—
48A	7·5	19·5	—	24·5	—	24·7	27·2	23·6	—	22·2	—	12·3

These results indicate that the optimum concentration varies slightly for different samples of yeast, but that it approximates to molar; moreover, very

slight difference is observable in the effect of concentrations ranging from 0·7 to 1·1 molar.

Experiments made with other salts showed that the phenomenon described for sodium chloride is a general one for all salts, both of inorganic and organic acids. The following salts were all found to give positive results: Chlorides of sodium, potassium, lithium, ammonium, magnesium, calcium, and barium; sulphates of sodium, potassium, ammonium, and magnesium; sodium salts of phosphoric, hexosephosphoric, arsenic, acetic, malic, citric, lactic, pyruvic, and glyceric acids.

With the salts of organic acids, the possibility exists that these may themselves be the source of the carbon dioxide. Neubauer (3) and Neuberg, Hildesheimer, Tir, and Karczag (4, 5, 6) have, in fact, stated that some races of yeast are capable of producing carbon dioxide from salts of lactic, glyceric, pyruvic, oxalacetic, and many other acids. As this phenomenon is accompanied by the disappearance of the acid in question, it can readily be distinguished from that which forms the subject of the present paper.

2. *The Nature of the Effect Produced by Salts on the Autofermentation of Yeast.*

It seemed advisable at the outset to ascertain experimentally if the increase in the rate of gas production were actually due to stimulation of the glycogenase, as was to be expected, or of the zymase. The sugar fermentation of 1 grm. of yeast immersed in molar sodium chloride gave only 1·7 c.c. of carbon dioxide per five minutes, as against 4·1 c.c. in the case of a water control. The action of the zymase is therefore inhibited rather than enhanced by this treatment. The increase in the rate of autofermentation would accordingly seem to result from a more efficient working of the glycogenase.

This might be due to one or more of the following causes:—

(1) To some specific action of the salt employed.

(2) To a concentration within the cell by removal of water as a result of plasmolysis.

(3) To removal from the cell of some substance or complex which has an inhibitory or controlling action upon the rate of glycogen fermentation.

(4) To disorganisation of the cell, whereby the factor controlling the access of enzyme to glycogen is in some way modified.

(5) To "hormone" action of the substance on the lines suggested by H. E. and E. F. Armstrong.

(1) *Specific Action.*—In order that a specific action should be exerted, it is essential that the agent should be capable of entering the cell. As regards

this question, in an earlier work* the conclusion was reached that most salts are probably not capable of penetrating beyond the outer layers of the cytoplasm. This would render any specific action upon the enzyme very doubtful. Moreover, it is improbable that so many different salts should exert a similar effect. Further, such action, if exerted in the cell, should also be exhibited in the contents after removal from the cell. The following table shows the result of addition of salt to yeast-juice both in presence and absence of added sugar :—

Table III.—Effect of Sodium Chloride upon Fermentation by Yeast-juice.

	Cubic centimetres of carbon dioxide evolved by 25 c.c. of yeast-juice in 18 hours.				
	Control.	+ 0·14 grm. NaCl.	+ 0·36 grm. NaCl.	+ 0·72 grm. NaCl.	+ 1·45 grm. NaCl.*
Sugar free.....	35·3	28·0	18·2	8·2	2·2
+ 1 grm. glucose...	55·9	42·2	29·8	14·5	3·1

* Molar concentration.

These numbers prove that the autofermentation is diminished in practically the same proportion as the sugar fermentation, and they afford no evidence of acceleration of the action of the glycogenase.

Very similar results were obtained with zymin.

Table IV.—Effect of Sodium Chloride upon Fermentation by Zymin.

	Cubic centimetres of carbon dioxide evolved by 5 grm. zymin + 20 c.c. solution in 5 hours.			
	Water.	M/10 NaCl.	M/4 NaCl.	M/2 NaCl.
Sugar free	77·2	64·0	51·7	32·4
+ 1 grm. glucose	173·2	162·4	136·2	83·5

It follows from these experiments that the direct action of salt upon the enzymes of yeast is that of an inhibitant, and that the acceleration of the autofermentation of yeast by salt cannot be due to a specific effect of the latter. This, however does not exclude the possibility that certain substances which accelerate the action of yeast-juice and zymin may also exert a specific effect upon the autofermentation.

(2) *Plasmolysis of the Cell.*—It has been demonstrated by Paine that with molar concentration of sodium chloride strong plasmolysis occurs, while decimolar solution produces no such result. The effect of these concentrations upon the autofermentation of yeast is shown in the following table :—

* Paine, *loc. cit.*

Table V.—Showing Effect of Molar and Decimolar Solutions of Sodium Chloride.

Time, in hours.	Cubic centimetres of carbon dioxide evolved from 5 grm. yeast and 20 c.c. of solution.		
	NaCl, molar.	NaCl, decimolar.	Water control.
1	37·5	11·2	12·6
2	57·4	17·4	18·2
3	66·6	21·4	21·5
4	70·8	23·9	23·8
5	71·7	26·1	26·1

It follows that sodium chloride solution is without influence upon the autofermentation when the concentration is so low as to produce no plasmolysis of the yeast.

Experiments were, therefore, made to determine the effect of iso-osmotic solutions of various substances, which had all been found to produce plasmolysis in a similar manner to sodium chloride. The osmotic coefficients were taken from the tables given in Pfeffer's 'Physiology of Plants,' and in some cases the freezing points of the solutions were determined. The results are given in the following tables :—

Table VI.—Effect of Iso-osmotic Solutions of Salts.

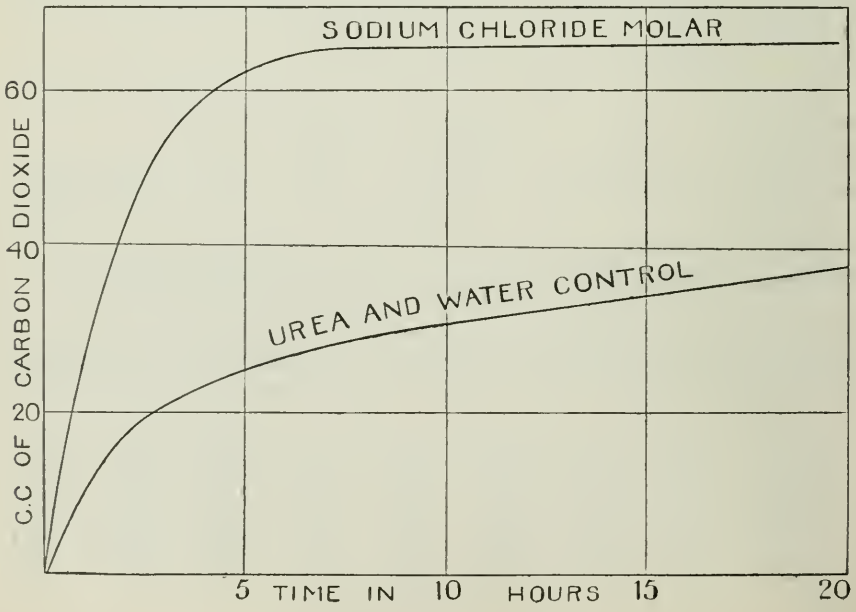
No. of expt.....	82. A.	B.	C.	D.	83. A.	B.	C.	D.
Details.....	10 grm. pressed yeast + 20 c.c. of solution.							
Substance employed	NaCl	K_2HPO_4	$CaCl_2$	Water control	NaCl	K_2SO_4	Mannitol	Water control
Concentration ...	Molar	13 grm. 100 c.c.	8·3 grm. 100 c.c.	—	½ molar	6·5 grm. 100 c.c.	13·5 grm. 100 c.c.	—
Time, in hours.	Cubic centimetres of carbon dioxide.				Cubic centimetres of carbon dioxide.			
0	—	—	—	—	—	—	—	—
0·5	18·3	19·6	19·5	10·5	14·6	13·3	12·7	10·5
1·0	31·8	33·3	33·5	16·4	24·0	22·0	21·0	16·4
1·5	42·1	42·7	43·3	20·9	30·8	28·0	28·2	20·9
2·0	49·3	49·3	50·5	24·8	36·0	32·7	31·8	24·8
2·5	53·6	53·3	54·9	27·8	39·2	35·5	34·6	27·8
3·0	57·3	56·5	58·5	30·8	41·7	38·0	36·8	30·8
4·5	62·0	61·2	64·3	36·8	45·7	43·0	41·5	36·8

Table VII.—Effect of Solutions Iso-osmotic with 0·5 Molar Potassium Nitrate.

No. 84	10 grm. pressed yeast + 20 c.c. solution.					
Substance.....	KNO_3	$MgSO_4$	$BaCl_2$	Mannitol.	Glycerol.	Water.
Concentration.....	5·05 grm. 100 c.c.	18·45 grm. 100 c.c.	9·1 grm. 100 c.c.	13·65 grm. 100 c.c.	6·95 grm. 100 c.c.	—
Depression of freezing-point	1·46°	1·51°	1·80°	1·57°	1·52°	—
Time.	Cubic centimetres of carbon dioxide.					
12.45	—	—	—	—	—	—
1.15	29·5	27·0	30·1	29·2	27·6	19·0
1.45	54·3	50·0	57·8	56·4	50·0	31·6
2.15	75·8	70·0	80·9	78·5	66·6	41·0

These experiments point very strongly to the removal of water from the cell as the essential factor, since it is seen that, when substances which cause plasmolysis are employed, solutions of equal osmotic pressure produce an equal degree of acceleration.

In order to obtain convincing proof of this, it was necessary to find some



substance which would produce no plasmolysis of yeast even in concentrated solution, and to show that it would not cause acceleration. In earlier experiments urea was found to produce no plasmolysis at molar concentration. The determination of the effect of this substance upon the rate of autofermentation was therefore of first importance. In one experiment molar urea was compared with molar sodium chloride and water. The urea was found to be without influence, as shown in the curves (p. 453).

In the following experiment (No. 85) the effects of isotonic solutions of urea, sodium chloride, and potassium nitrate were compared.

Table VIII.—Effect of Urea Solutions.

No. 85.	10 grm. yeast + 20 c.c. solution.				
	NaCl	Urea	KNO_3	Urea	Water
Concentration.....	5·85 grm. 100 c.c. = molar	9·0 grm. 100 c.c.	5·05 grm. 100 c.c. = 0·5 molar	4·5 100 c.c.	—
Depression of freezing point	—	—	1·46°	1·42°	—
Time.	Cubic centimetres of carbon dioxide.				
0·0	—	—	—	—	—
0·5	34·0	17·0	21·3	16·5	17·0
1·0	65·0	29·2	46·7	29·4	29·3
1·5	86·5	34·5	60·3	35·5	35·5

Urea is thus seen to be without influence upon the rate of autofermentation, although, as shown by the depression of the freezing-point, the solutions of this substance were isotonic with the corresponding salt controls. The fact that plasmolysis of the cells is not produced by urea solutions would seem to indicate that this substance can penetrate freely through the cytoplasm of the yeast cell. An experiment was made to investigate this point, the method described by Paine (2) being employed; 100 grm. of yeast were suspended in 100 grm. of molar urea solution, allowed to stand 20 hours at a temperature approximating to zero, and the distribution of urea determined (Table IX).

Urea is thus seen to penetrate readily into the cells, the factor K representing the coefficient of diffusion being of the same order as that obtained for alcohol, namely, 0·85 to 0·87. Although urea enters the cells it is without influence upon the rate of autofermentation.

Table IX.—Showing Diffusion of Urea into the Yeast-cell.

	Initial yeast.	Initial liquid.	Final yeast.	Final liquid.	P = grm. urea per 100 grm. water within the cells.	P_1 = grm. urea per 100 grm. water outside the cells.	$K = P/P_1$.
	grm.	grm.	grm.	grm.			
Solids other than urea	32·50	—	31·61	0·85			
Urea	—	5·94	2·50	3·45			
Water	67·50	94·06	72·49	89·10			
Total weight	100·00	100·00	106·60	93·40	3·43	3·87	0·89

Removal of Water by Partial Drying.—If the acceleration of the enzymic activity were due simply to concentration within the cell, removal of water by drying would be expected to produce the same result as removal of water by plasmolysis. In order to investigate this 10 grm. of pressed yeast which had been passed through a 3 mm. sieve were placed in a fermentation flask and subjected to a current of air for 20 minutes. This flask and a control were then connected with the gas-measuring apparatus and warmed in the water-bath at 25°. The rate of autofermentation was considerably increased by this simple method of removing water.

Table X.—Effect of Partial Drying by Air.

Time, in mins.	Cubic centimetres of carbon dioxide given by 10 grm. yeast.	
	After 20 minutes blow.	Control.
15	14·5	4·3
30	27·5	8·9
45	36·4	13·3
65	43·6	18·4
85	47·8	23·4

In another experiment three lots of 10 grm. of pressed yeast were weighed out, of which B and C were dried in a vacuum desiccator for two and four hours respectively, whereby B lost 2 grm. and C 3·2 grm. of water. The rate of autofermentation of these samples was compared against A as control.

Table XI.—Effect of Partial Desiccation *in Vacuo*.

Time.	Cubic centimetres of carbon dioxide yielded per hour by—		
	A. 10 grm. yeast. Control.	B. 10 grm. yeast dried 2 hours.	C. 10 grm. yeast dried 4 hours.
1st hour	36·0	46·1	55·5
2nd „	15·3	17·2	39·3
3rd „	14·1	14·7	38·7
4th „	12·9	14·8	32·4
5th „	13·7	14·7	22·4
24 hours (total)	281·6	248·3	234·9

In this experiment a loss of 3·2 grm. of water from 10 grm. of yeast, equal to approximately half the water content of the cells, had the effect of more than doubling the rate of autofermentation.

(3) The possibility of the removal from the cell of some inhibitory or controlling substance during plasmolysis is negatived by these last experiments, wherein the increase of autofermentation was produced under conditions which render such removal impossible unless the substance be a volatile liquid.

(4) The disorganisation of the cell, possibly by the disintegration of a material membrane or network, has been adduced as the cause of some of the effects of anæsthetics on the living cell [Overton (7), Lepeschkin (8), Hans Meyer (9)], and it is not impossible that in certain instances this phenomenon plays some part in the acceleration of the autofermentation of yeast. This possibility is specially present in the case of a substance like toluene, which exerts an anæsthetic effect upon yeast.

The following experiment is typical of many; 10 grm. yeast were mixed with (*a*) 25 c.c. water, (*b*) 25 c.c. water and 5 c.c. toluene, well shaken, and incubated at 25°:—

Table XII.—Effect of Toluene on the Autofermentation of Yeast.

Time.	Cubic centimetres of carbon dioxide.			
	a. Water.		*b*. Water + toluene.	
	Total.	Rate per 10 mins.	Total.	Rate per 10 mins.
10 mins.	3	3	8·5	8·5
20 „	5·3	2·3	15·5	7·0
30 „	7·6	2·3	22·7	7·2
40 „	9·4	1·6	29	6·3
50 „	11·3	1·9	35·9	6·9
3 hrs.	21·5	—	86·1	—

Other instances of the same effect are the following, all of which refer to 10 grm. of yeast:—

Table XIII.—Effect of Toluene.

Date.	Time, in hours.	Water alone.	Water + toluene.
3.2.08	1	4·2	29·5
10.9.09	2·5	6·3	30·4
17.9.09	2	6·9	34·5
17.10.07	4	48·9	80·7
20.10.07	5	28	97·6

As in the case of salt solutions the rate slowed down comparatively soon, owing to exhaustion of the fermentable material. The effect is not due to a specific action on the enzymes, since toluene has either no effect or a slight inhibitory effect on the autofermentation of yeast-juice, as is shown by the following result: 25 c.c. of yeast-juice in three hours gave 40·3 c.c. of CO_2; in presence of 5 c.c. of toluene the same volume of yeast-juice gave 34 c.c.

It is, however, not impossible that this result may be explicable on the ground of plasmolysis. In spite of the small solubility of toluene in water a considerable degree of plasmolysis is observed when yeast is shaken with water and excess of toluene. Further experiments on this point are in progress.

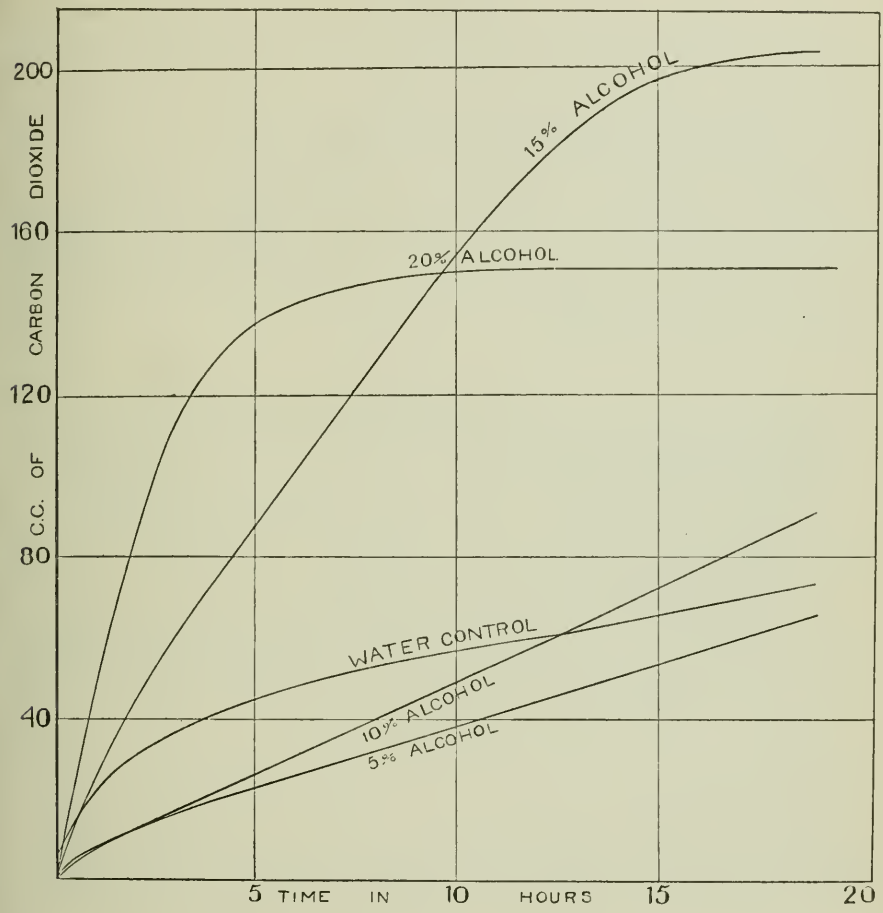
(5) With regard to the possibility that the foregoing changes may be ultimately due to the action of hormones in the manner suggested by H. E. and E. F. Armstrong (7) no very definite conclusion can be drawn. The action of toluene on yeast undoubtedly presents the closest analogy to that which it exerts on the Aucuba leaf, and it cannot be denied that the various salts employed do penetrate at all events into the outer layers of the yeast cell. Several of the phenomena, however, appeared to be difficult to explain in this way, especially the lack of action of a substance like urea, which penetrates the cell, and the causation of the phenomenon by simple drying. In any case the acceleration caused by salts is accompanied by concentration of the cell contents, so that dilution cannot in these instances be the effective cause, as suggested by Armstrong* for the phenomenon observed by him.

3. *Effect of Alcohol on Autofermentation.*

The plasmolysing effect on yeast of solutions of alcohol was found to be practically absent from concentrations up to 10 per cent. (rather more than

* *Loc. cit.*

twice molar), but above this concentration plasmolysis became well marked. The influence of alcohol solutions on the autofermentation of yeast is shown by the following curves, which apply to 10 grm. of yeast:—



Concentrations of alcohol which plasmolyse the cells produce a considerable increase in the rate of autofermentation. With 20 per cent. the action of the enzyme almost came to an end after about seven hours, at which time 147 c.c. of gas had been collected as against 52 c.c. from the water control. The weaker concentrations of alcohol at first produced an inhibitory effect upon the rate. After a short time, however, the rate increased, and then slightly exceeded that of the water control.

Eventually, after eight days, the volume of gas yielded from each, with the exception of that in presence of 20 per cent. alcohol, was practically identical and approximately equal to 200 c.c.

The behaviour of alcohol, therefore, is in accord with that of urea, although the effect is not quite so simple.

Summary.

1. All dissolved substances which plasmolyse the yeast-cell also cause a large increase in the rate of autofermentation.

2. Substances such as urea, which even in concentrated solution do not produce plasmolysis, have no accelerating effect.

3. Toluene produces a similar effect to concentrated salt solutions.

4. The effect produced by salts is probably a direct result of the concentration of the cell contents due to plasmolysis, but in the case of toluene it is possible that some other factor (such as disorganisation of the cell, or hormone action) is concerned.

REFERENCES.

1. Harden, Thompson, and Young, 'Biochem. Journ.,' 1910, vol. 5, p. 230.
2. Paine, 'Roy. Soc. Proc.' (in press).
3. Neubauer and Fromherz, 'Zeitschr. f. Physiol. Chem.,' 1911, vol. 70, p. 326.
4. Neuberg and Hildesheimer, 'Biochem. Zeitschr.,' 1911, vol. 31, p. 170.
5. Neuberg and Tir, *ibid.*, 1911, vol. 32, p. 323.
6. Neuberg and Karczag, *ibid.*, 1911, vol. 36, p. 60.
7. Overton. 'Jahrb. f. Wiss. Bot.,' 1900, vol. 34, p. 670.
8. Lepeschkin, 'Ber. Deutsch. Bot. Ges.,' 1911, vol. 28, p. 383, and vol. 29, p. 349.
9. Meyer, Hans, 'Report Int. Cong. Applied Chemistry,' 1909, IVa, vol. 2, p. 37.
10. Armstrong, H. E. and E. F., 'Roy. Soc. Proc.,' B, 1910, vol. 82, p. 588.

Harrison and Sons, Printers in Ordinary to His Majesty, St. Martin's Lane.

Sonderabdruck aus d. Biochemischen Zeitschrift 40. Bd., S. 458.

Der Mechanismus der alkoholischen Gärung.

Von

Arthur Harden und **William J. Young.**

(Aus der Biochemischen Abteilung des Lister Instituts, London.)

(Eingegangen am 11. März 1912.)

Mit 3 Figuren im Text.

In einer Anzahl neuerer Arbeiten hat A. v. Lebedew[1]) eine Theorie der alkoholischen Gärung aufgestellt, nach welcher eine Hexose in Kohlensäure und Alkohol in der folgenden Weise zerlegt wird:

1. $C_6H_{12}O_6 = 2C_3H_6O_3$
2. $2C_3H_6O_3 + 2RHPO_4 = 2C_3H_5O_2RPO_4 + 2H_2O$
3. $2C_3H_5O_2RPO_4 = C_6H_{10}O_4(RPO_4)_2$
4. $C_6H_{10}O_4(RPO_4)_2 + H_2O = C_2H_5OH + CO_2 + C_3H_5O_2RPO_4 + RHPO_4$
5. $C_6H_{10}O_4(RPO_4)_2 + 2H_2O = 2C_2H_5OH + 2CO_2 + 2RHPO_4$

Diese Theorie stützt sich auf die Tatsache, daß der durch Macerierung mit Wasser von lufttrockener Hefe dargestellte Hefesaft Dioxyaceton prompt vergärt und bei Gegenwart von Phosphat in ein Hexosephosphat überführt, das mit dem aus Glucose, Fructose oder Mannose unter gleichen Bedingungen gewonnenen identisch ist. Dies ist eine Modifikation der früher von Iwanoff[2]) formulierten Hypothese, die er auf Grund seiner Untersuchungen mit Zymin und Hefanol entwickelte, daß bei der Hefegärung von Hexosen der Zucker depolymerisiert

[1]) A. Lebedew, Compt. rend. 153, 136, 1911; Bull. Soc. Chim. 9, 678, 1911; Ber. d. Deutsch. chem. Ges. 44, 2932, 1911.

[2]) Iwanoff, Centralbl. f. Bakt., Abt. II, 24, 1, 1909.

wird. Die dabei entstandenen Produkte bilden dann bei Reaktion mit Phosphorsäure ein Triosephosphat, aus welchem Kohlensäure, Alkohol und freies Phosphat entstehen. Seitdem ist nachgewiesen worden, daß die phosphororganische Verbindung ein Hexose-diphosphorester ist, aber hiervon abgesehen haben Harden und Young[1]) gezeigt, daß Iwanoffs Theorie den Tatsachen widerspricht, und die damals erbrachten Beweisgründe können ebenso zur Entkräftung der von Lebedew modifizierten Theorie dienen. Auf die Gärung des Dioxyacetons wird später eingegangen werden. Diese beiden Theorien haben das Gemeinsame, daß die Hexosephosphorsäure, Iwanoffs Triosephosphorsäure, als ein der Bildung von Alkohol und Kohlensäure vorangehendes Zwischenprodukt angesehen wird.

Die Verfasser haben wiederholt gezeigt, daß durch Phosphatzusatz zu einem Gemisch von Hefesaft oder Zymin und einer Hexose, die mit konstanter Geschwindigkeit gärt, eine auffallende Beschleunigung in der Gärung auftritt, bis ein Maximum erreicht wird. Diese erhöhte Geschwindigkeit dauert kurze Zeit an, fällt plötzlich ab und kehrt zu ihrem ursprünglichen Wert zurück. Die Kohlensäure- und Alkoholmengen, die in dieser Zeit im Überschuß gegen die in Abwesenheit von hinzugefügtem Phosphat auftretenden gebildet werden, sind chemisch diesem letzteren nach folgender Gleichung äquivalent:

$$(1) \qquad 2C_6H_{12}O_6 + 2Na_2HPO_4 = 2CO_2 + 2C_2H_6O + C_6H_{10}O_4(PO_4Na_2)_2 + 2H_2O.$$

Nach dieser Periode findet man überdies das ganze Phosphorsalz als Hexosephosphat vor. Die Bildung von Hexosephosphat wird, wie man hieraus sieht, von einer alkoholischen Gärung begleitet, deren Umfang ganz genau der Menge des hinzugesetzten Phosphats entspricht. Die Gärungsgeschwindigkeit nimmt rapid ab, sobald alles freie Phosphat in Hexosephosphat umgeformt ist, obgleich die Konzentration des letzteren gerade dann auf der Höhe ist. Wenn die Bildung von Hexosephosphat derjenigen der Kohlensäure und des Alkohols voranginge, müßte das Umgekehrte eintreffen — die Größe der alkoholischen Gärung müßte sich dann proportional zu derjenigen des gespaltenen Hexosephosphats verhalten, wäh-

[1]) Harden und Young, Centralbl. f. Bakt., Abt. II, 26, 179, 1910.

rend die Gärungsgeschwindigkeit des Hexosephosphats von seiner Konzentration abhängen und am größten sein müßte, wenn letztere den höchsten Grad erreicht hat.

Die Übereinstimmung in der zugefügten Phosphat- und der darauf gebildeten Gärungsmenge ganz außer acht lassend, sucht Lebedew[1]) diese Anomalie dadurch zu erklären, daß das Hexosephosphat selbst die Gärungswirkung hemmt, sobald seine Konzentration eine gewisse Grenze überschritten hat. Daß hierin unmöglich der Grund in der Geschwindigkeitsabnahme unter den obwaltenden Versuchsbedingungen liegen kann, geht klar aus der Tatsache hervor, daß sich bei Zusatz von mehr Phosphat die Erscheinung wiederholt. Die Gärungsgeschwindigkeit steigt wieder an, bis ein Extraquantum Kohlensäure und Alkohol, jener neuen Phosphatmenge entsprechend, gebildet worden ist, fällt dann plötzlich auf denselben konstanten Wert wie im Anfang, und alles Phosphat ist dann als Hexosephosphat zugegen. Daher entsprechen Lebedews Gleichungen in dieser Hinsicht nicht den aus Experimenten gewonnenen Resultaten und können folglich nicht als gültig angesehen werden. Auf das aus unseren Versuchen gesammelte Beweismaterial bezüglich dieser Phänomene haben wir verschiedentlich hingewiesen. Zur Orientierung über die Versuche, welche die obigen Punkte behandeln, vergleiche man (für Hefesaft) Proc. Roy. Soc. B. 77, 415, 1906 und (für Zymin) Centralbl. f. Bakt., Abt. II, 26, 181, 1910.

Reaktion des Lebedewschen Macerationssaftes mit Phosphat.

Der nach der Lebedewschen Methode, Maceration von getrockneter Hefe, dargestellte Saft zeigt dasselbe Verhalten, wie aus den folgenden Versuchen ersichtlich ist.

Es kam die von Schroder in München hergestellte und von Lebedew[2]) benutzte Trockenhefe zur Anwendung. Maceration und Filtration wurden genau nach den Vorschriften des letzteren ausgeführt.

Die Gärungsversuche wurden bei 25° in Gegenwart von Toluol in dem schon beschriebenen Apparat[3]) angestellt, so daß Messungen der

[1]) Lebedew, Ber. d. Deutsch. chem. Ges. 44, 2938, 1911.
[2]) Lebedew, Ber. d. Deutsch. chem. Ges. 44, 2935, 1911.
[3]) Harden, Thompson und Young, Bioch. Journ. 5, 230, 1910.

Gärungsmengen jede 5 Minuten vorgenommen werden konnten. Alle Flüssigkeiten wurden vor Beginn jedes Versuchs mit CO_2 gesättigt und die Gärkolben vor jeder Ablesung tüchtig geschüttelt[1]).

Das freie Phosphat wurde mittels Fällung mit Magnesiumcitratgemisch bestimmt und als $Mg_2P_2O_7$ gewogen. Der durch Maceration von Trockenhefe dargestellte Hefesaft enthält ungebundenes Phosphat, so daß bei Hinzufügung von Zucker ein hoher Anfangswert der Gärung erzielt wird, der, sobald dieses Phosphat in Hexosephosphat übergeführt ist, auf einen konstanten Wert heruntergeht. Das während dieser Periode über die der konstanten Geschwindigkeit entsprechende Menge gebildete Kohlendioxyd entspricht ganz genau dem freien, im Saft vorhandenen Phosphat. Wenn nach Ablauf dieser Zeit ein neues Quantum Phosphat zugesetzt wird, wiederholt sich derselbe Vorgang. Alle diese Momente kommen in dem im folgenden beschriebenen Versuch deutlich zur Geltung, wo drei Proben mit je 20 ccm Macerationssaft im Gärschrank 20 Minuten lang angesetzt wurden.

Nr. 1 wurde dann gekocht, abfiltriert und das freie Phosphat im Filtrat bestimmt. Nr. 2 und 3 wurden mit je 5 ccm einer Lösung von 2 g Fructose versetzt, die vorher bei 25° mit CO_2 gesättigt worden war. Die Gärungsmengen wurden hierauf beobachtet und in der folgenden Tabelle zusammengestellt.

Tabelle I.

Zeit	ccm CO_2 in den vorhergehenden 5 Minuten		Gesamtmenge	
Minuten	2	3	2	3
5	2,5	2,3	2,5	2,3
10	8,3	9,2	10,8	11,5
15	19,7	22,9	30,5	34,4
20	30,2	31,2	60,7	65,6
25	19,3	18,0	80,0	83,6
30	4,9	5,3	84,9	88,9
35	5,0	5,0	89,9	93,9
40	5,0	5,0	94,9	98,9

Nach 40 Minuten, als die entwickelte CO_2-Menge den konstanten Wert von 5 ccm per 5 Minuten erreicht hatte, wurde die Gärung in Nr. 2 unterbrochen, indem man den Kolben in kochendes Wasser tauchte. Die Lösung wurde filtriert und das freie Phosphat im Filtrat bestimmt.

[1]) Es ist von wesentlicher Bedeutung, daß die Gärlösungen vor jeder Beobachtung gehörig geschüttelt werden, da sowohl Preß- wie Macerationssaft sich leicht mit CO_2 übersättigen. In einem Fall z. B. wurden aus einem gärenden Gemisch, 100 ccm Macerationssaft und 10 g Glucose enthaltend, das 10 Stunden lang ohne Schütteln gestanden hatte, nur 231 ccm CO_2 in dieser Zeit entwickelt. Nach Schütteln entwickelte sich jedoch sofort noch ein Plus von 452 ccm CO_2.

Freies Phosphat in den ursprünglichen 20 ccm = 0,3129 g $Mg_2P_2O_7$
„ „ nach 40 Minuten . . . . . = 0,0420 g „

Verestert . . . . . 0,2709 g $Mg_2P_2O_7$
entsprechend 54,8 ccm CO_2 bei normalem Druck und Temperatur ($Mg_2P_2O_7 = 2CO_2$).

	Nr. 2	Nr. 3
Gesamtmenge von CO_2 in 40 Minuten . .	= 94,9 ccm	98,9 ccm
Korrektur für konstanten Wert = 5 ccm in 5 Minuten	= 40 „	40 „
Entwickelte CO_2 bei 15,2° und 761,2 mm Druck auf Grund des freien Phosphats	= 54,9 ccm	58,9 ccm
Bei normaler Temperatur und Druck . .	= 51,2 ccm	54,9 ccm

Dem Kolben Nr. 3 wurden nach diesen 40 Minuten 5 ccm einer annähernd 0,3 molekularen Lösung Na_2HPO_4 zugesetzt, die Fructose enthielt und bei 25° mit CO_2 gesättigt wurde. Alle 5 Minuten Ablesungen wie vorher.

Tabelle II.

Zeit nach Hinzufügung von Phosphat Minuten	ccm CO_2 in den vorhergehenden 5 Minuten	Gesamtmenge
5	15,2	15,2
10	14,8	30,0
15	14,6	44,6
20	12,0	56,6
25	5,9	62,5
30	5,1	67,6
35	4,9	72,5
40	5,0	77,5

Nach Ablauf dieser Zeit wurde das Gemisch gekocht, abfiltriert und das freie Phosphat wie vorher im Filtrat bestimmt.

Hinzugefügtes Phosphat . . . . . = 0,1713 g $Mg_2P_2O_7$
Ursprüngliche Menge Phosphat im Hefesaft . = 0,0420 g „

Gesamtmenge . . . . . = 0,2133 g $Mg_2P_2O_7$
Freie Phosphatmenge am Schluß . . . . . = 0,0507 g „

Verestert . . . . . = 0,1626 g $Mg_2P_2O_7$
entsprechend 32,9 ccm CO_2 bei normalem Druck und Temperatur.

Gesamte in 40 Minuten entwickelte CO_2-Menge . . . = 77,5 ccm
Korrektur für den Wert bei Abwesenheit von Phosphat = 5×8 . . . . . = 40,0 „

Durch Phosphatzusatz entwickelte CO_2-Menge . . . . = 37,5 ccm (15,2° und 761,2 mm).

Bei normalem Druck und Temperatur . . . . . = 34,98 ccm

Aus diesen Zahlen geht deutlich hervor, daß das rapide Sinken der Gärungsintensität auf einen konstanten Wert nicht

auf der hemmenden, durch Anhäufung von Hexosephosphat ausgeübten Wirkung auf den Gärungsprozeß beruht, sondern auf dem Verschwinden von freien Phosphaten aus der Lösung infolge deren Umwandlung in Hexosephosphat. Dieser Versuch zeigt auch, daß das ungebundene Phosphat der Kohlensäure, welche infolge seines Vorhandenseins entwickelt wird, vollständig äquivalent ist.

v. Lebedews Gleichungen sind mithin, soweit sie die CO_2- und Alkoholbildung als derjenigen von Hexosephosphat folgend anstatt sie begleitend darstellen, ungenau.

Vergleich der Gärungsmengen von Hexosephosphat allein und in Gegenwart von Zucker.

Nach der schon früher geäußerten Ansicht der Autoren[1]) wird Hexosephosphat durch ein im Hefesaft vorhandenes Enzym (Hexosephosphatase) in Hexose und freies Phosphat gespalten.

$$(2) \qquad C_6H_{10}O_4(PO_4R_2)_2 + 2H_2O = C_6H_{12}O_6 + 2R_2HPO_4.$$

Letzteres tritt dann wieder bei vermehrten Zuckermengen in die oben (1) angegebene Reaktion. Die konstante Gärungsmenge, die bei einem Überschuß von Zucker nach Verwandlung des gesamten Phosphats in Hexosephosphat sich ergibt, wird auf diese Weise durch die Geschwindigkeit bestimmt, bei der das Phosphat gemäß der Gleichung (2) in Freiheit gesetzt wird. Daraus folgt, daß wenn man Hefesaft auf Hexosephosphat bei Abwesenheit von Zucker einwirken läßt, die Gärungsprodukte geringer als bei dessen Anwesenheit sein müßten, da die durch Hydrolyse von Hexosephosphat entstandene Zuckermenge nur halb so groß ist, wie sie zur Reaktion mit dem in derselben Zeit freigewordenen Phosphat erforderlich ist.

Nach Lebedews Theorie müßte die anfängliche Gärungsgeschwindigkeit des Hexosephosphats bei Ab- wie Anwesenheit des Zuckers gleich groß sein, da das Hexosephosphat unmittelbar der Bildung von Kohlensäure und Alkohol vorangeht. In den folgenden drei Versuchen mit Macerationssäften aus drei Proben von Schroders Hefe werden diese Werte verglichen. Das Hexosephosphat wurde im Saft vorbereitet, indem man einen

[1]) Harden und Young, Proc. Roy. Soc. B. 80, 299, 1908.

geringen Überschuß von dem, zur Umwandlung des gesamten freien, im Saft vorhandenen Phosphats in Hexosephosphat notwendigen Zucker hinzufügte. Wenn dieser Punkt erreicht wurde, fiel die Gärungsmenge auf den konstanten Wert herab, blieb eine Zeit lang im Verhältnis zu dem überschüssigen Zucker auf dieser Stufe und sank dann auf den niedrigeren Hexosephosphatwert, in dem Maße, wie sich der im Übermaß vorhandene Zucker erschöpfte. Fügte man in diesem Augenblick neuen Zucker hinzu, so wurde ein sofortiges Zurückgehen auf den höheren konstanten Wert beobachtet.

1. Versuch. Zwei Gemische mit je 20 ccm Macerationssaft, 0,5 g Fructose enthaltend, wurden im Brutschrank angesetzt und ihre Gärung beobachtet. Es wurde ein Maximum von 28 ccm pro 5 Minuten erreicht, das nach 25 Minuten auf 5 ccm und nach 35 Minuten auf 1,5 ccm per 5 Minuten herabsank. Eine dritte Probe von 20 ccm, der man 2 g überschüssigen Zucker hinzugefügt hatte, ergab denselben hohen Maximalwert, jedoch fiel dieser nur auf 5 ccm in 5 Minuten und blieb damit konstant.

Nach 35 Minuten versetzte man die erste Probe mit 0,5 g Fructose, die zweite dagegen nicht — die Gärungen hielten an. Die Ergebnisse erhellen aus der Tabelle III (1).

2. Versuch. 90 ccm Hefesaft wurden mit 5 g Fructose und 15 ccm einer 0,3 molaren Lösung Na_2HPO_4 im Thermostat angesetzt, bis alles freies Phosphat gebunden und der Überschuß an Zucker aufgebraucht war. Aus diesem Gemisch wurden zwei Portionen von je 20 ccm entnommen, die eine wurde mit 5 ccm Wasser, die andere mit 5 ccm, 1 g Fructose enthaltend, versetzt. Die Resultate sind in Tabelle III (2) zusammengestellt.

Tabelle III.

Zeit nach Zusatz Minuten	ccm CO_2 in vorhergehenden 5 Minuten			
	mit Fructose (1)	ohne Fructose (1)	mit Fructose (2)	ohne Fructose (2)
5	13,4	1,5	11,6	1,7
10	5,6	1,2	5,6	1,4
15	4,9	1,3	5,0	1,6
20	5,3	1,4	5,1	1,4
25	5,3	1,3	4,7	1,6
40	4,8	1,4	4,4	1,8
45	5,0	1,4	4,5	1,7

3. Versuch. Drei Portionen von je 15 ccm Macerationssaft wurden mit 0,5 g Glucose wie vorher im Brutschrank zur Gärung angesetzt. In jeder Probe wurde ein Maximum von 19 ccm per 5 Minuten erreicht, welches auf 4 ccm herunterging und auf diesem Werte 20 Minuten lang

sich erhielt, um dann aus Mangel an Zucker eine Tendenz zur weiteren Abnahme zu zeigen. Zu dieser Zeit wurden zugesetzt:

Zu (1) 10 ccm neutralisierte Lösung von Hexosephosphorsäure (8,5 g Säure in 100 ccm);

zu (2) 15 ccm der obigen Hexosephosphatlösung;

zu (3) 10 ccm Hexosephosphat + 0,5 g Glucose.

Alle drei Gemische wurden auf dasselbe Volumen mit Wasser aufgefüllt. Die Gärmengen sind in Tabelle IV angegeben.

Tabelle IV.

Zeit nach Zusatz	ccm CO_2 in vorhergehenden 5 Minuten		
Minuten	1.	2.	3.
5	2,9	2,5	9,7
10	2,3	2,2	3,8
15	2,0	1,9	3,7
20	1,6	1,6	4,3
25	1,6	1,7	3,7
30	1,5	1,5	3,8
35	1,5	1,5	3,8

Aus der Tatsache, daß in diesem Versuch 15 ccm Hexosephosphat dieselbe Menge wie 10 ccm ergaben, geht hervor, daß diese Substanz in dem Gemisch überschüssig vorhanden war.

Wir ersehen aus all diesen Versuchen, daß die aus Hexosephosphat allein entwickelte Kohlensäuremenge geringer ist, als wenn die Lösung auch Zucker enthält. Diese Versuche stimmen also mit den Ansichten der Autoren überein, widersprechen dagegen denjenigen Lebedews.

Während die Gärung allein aus dem Hexosephosphat vor sich geht, muß eine Anhäufung von freiem Phosphat stattfinden, da durch die Hydrolyse des Hexosephosphats nur so viel Zucker gebildet wird, als zur Reaktion mit der halben Menge in Freiheit gesetzten Phosphats erforderlich ist. In den oben angeführten Versuchen war eine geringe Ansammlung zustande gekommen, wie aus den hohen Werten in den Tabellen unmittelbar nach Zuckerzusatz ersichtlich ist, die fast sofort wieder auf den konstanten Wert heruntergingen. Unter diesen Bedingungen könnte man erwarten, daß die Gärungsmenge genau die Hälfte derjenigen beim Vorhandensein überschüssigen Zuckers betragen würde. Die wirklich ermittelten Werte stehen aber unter der Hälfte des Zuckerwertes. Man kann diese Unstimmigkeit wahrscheinlich durch die Bildung von höheren

Kohlenhydraten erklären, die bekanntlich in der Flüssigkeit stattfindet[1]), und durch die hemmende Einwirkung des nach der Hydrolyse von Hexosephosphat sich anhäufenden freien Phosphats[2]).

Die Versuche von Euler und Ohlsén.

Euler und Ohlsén[3]) behaupten, einen Macerationsextrakt aus einer besonderen Hefeart dargestellt zu haben, welcher gar keine Wirkung auf Glucoselösung ausübt, bevor diese nicht erst teilweise durch lebende Hefe angegoren ist. Ein solcher Extrakt ist dann fähig, aus dieser so behandelten Glucose und Phosphat Hexosephosphat zu bilden, ohne zugleich Kohlensäure zu entwickeln. Sollte diese Beobachtung Bestätigung finden, so wird es wichtig sein festzustellen, ob das produzierte Hexosephosphat dem ganzen aufgebrauchten Zucker restlos entspricht, oder ob irgendeine andere Substanz, der Natur nach ein Zwischenprodukt zwischen Zucker einerseits und Alkohol und Kohlensäure andererseits, gleichzeitig gebildet wird. Mit anderen Worten, es besteht die Möglichkeit, erstens, daß die Gärung des Zuckers stufenweise vor sich geht: im ersten Stadium vollzieht sich synchron aus 2 Molekülen Zucker die Bildung von Hexosephosphat und einer anderen Substanz, die weiterhin im zweiten Stadium in Kohlensäure und Alkohol zersetzt wird, und zweitens, daß der von Euler und Ohlsén bereitete Saft nicht das zur vollständigen Spaltung notwendige Enzym enthält. Daß die Theorie der Autoren mit der Bildung eines solchen Zwischenproduktes nicht im Widerspruch steht, ist schon ausgeführt worden[4]).

Gärung von Dioxyaceton.

Als die Theorie, daß wir in der Milchsäure das Mittelglied bei der Zersetzung der Zuckerarten durch Hefe in Kohlensäure und Alkohol ansehen müssen, durch Slators[5]) Versuche un-

[1]) Harden und Young, Ber. d. Deutsch. chem. Ges. 37, 1052, 1904.

[2]) Harden und Young, Proc. Roy. Soc. 80, 304, 1908.

[3]) Euler und Ohlsén, diese Zeitschr. 37, 313, 1911; Zeitschr. f. physiol. Chem. 76, 468, 1912.

[4]) Harden und Young, Proc. Roy. Soc. B. 82, 329, 1910.

[5]) Slator, Journ. Chem. Soc. 89, 141, 1906; 93, 231, 1908; Ber. d. Deutsch. chem. Ges. 40, 123, 1907.

haltbar wurde, kamen Buchner und Meisenheimer auf ihrer weiteren Suche nach einer Zwischensubstanz darauf, die Wirkung von Hefe und Hefesaft auf Methylglyoxal, Glycerinaldehyd und Dioxyaceton zu erproben. Schon vorher war von G. Bertrand[1]) gezeigt worden, daß lebende Hefe die letztgenannte Substanz sehr langsam vergärt. Buchner und Meisenheimer[2]) fanden, daß Methylglyoxal durch lebende Hefe oder Hefesaft nicht in Gärung versetzt wird, daß aber sowohl Dioxyaceton wie Glycerinaldehyd durch Preßhefe langsam und durch Hefesaft sehr langsam vergoren werden. Wenn Hefesaft, der im Vakuum auf die Hälfte eingeengt war, mit gekochtem, ebenfalls auf die Hälfte seines Volumens konzentrierten Saft (Koenzym) versetzt wurde, so konnte die Mischung Dioxyaceton in $2\,^0/_0$iger Lösung fast ebenso restlos wie Glucose vergären. Buchner und Meisenheimer betrachteten deshalb diesen Stoff als ein möglicherweise intermediäres Produkt der alkoholischen Gärung. In jüngster Zeit hat v. Lebedew entdeckt, daß der durch Maceration von lufttrockener Hefe gewonnene Saft auch Dioxyaceton angreift, und berichtet, daß diese Substanz in Konzentrationen bis zu $5\,^0/_0$ so leicht wie Rohrzucker vergoren wird. Ferner hat er konstatiert, daß dieser Macerationssaft außerdem in einer Mischung von Dioxyaceton und Phosphat die Bildung eines organischen Phosphorsäureesters, der mit dem aus Hexosen unter denselben Bedingungen gewonnenen identisch ist, bewirken kann. Auf diese Versuche stützt sich seine Hypothese, die wir schon oben erörtert haben.

Die Beweisführung Slators betreffs der Milchsäure kann auch auf Dioxyaceton angewandt werden, so daß es, angenommen, diese Substanz ist ein Zwischenprodukt bei der Umbildung von Zucker zu Kohlensäure und Alkohol, mindestens so schnell wie Zucker vergoren werden müßte, vorausgesetzt, daß seine vorhandene Menge nicht den Gärungsvorgang hemmt. Die letzte Frage wird sofort erledigt, wenn man festgestellt hat, ob Zusatz von Dioxyaceton zu einer Zuckergärung die Gärintensität verringert.

[1]) Bertrand, Ann. Chim. Phys. (8) 3, 181, 1904.

[2]) Buchner und Meisenheimer, Ber. d. Deutsch. chem. Ges. 43, 1773, 1910.

Solch ein Vergleich ist vor kurzem für lebende Hefe von Slator[1]) angestellt worden. Die dabei ermittelten Ergebnisse führen den genannten Forscher zu dem Schluß, daß Dioxyaceton nicht direkt durch lebende Hefe vergoren wird und folglich kein intermediäres Produkt bei der Gärung der Zuckerarten sein kann.

Die Schlußfolgerungen, welche Buchner und Meisenheimer sowie v. Lebedew aus ihren Versuchen mit Hefepreß- und Macerationssaft in bezug auf die Gärfähigkeit von Dioxyaceton und Glucose (oder Rohrzucker) zogen, sind nicht stichhaltig, da ihre Beobachtungen nur in verhältnismäßig langen Zwischenräumen — 3 Stunden und mehr — gemacht wurden und deshalb kein präzises Bild von dem Gang der Erscheinungen geben. Wenn dagegen in Zeiträumen von einigen Minuten anstatt Stunden beobachtet wird, wie dies in den im folgenden beschriebenen Versuchen geschehen ist, so kommt sofort ein großer Unterschied in dem Gärprozeß in den beiden Fällen zum Vorschein.

Versuche mit Macerationssaft.

Der folgende Versuch ist typisch für mehrere, in welchen die Wirkung des Macerationssaftes, der nach der v. Lebedewschen Methode aus Schröderscher getrockneter Hefe hergestellt war, auf Rohrzucker, Dioxyaceton[2]) und ein Gemisch von beiden Substanzen verglichen wurde.

Jedesmal wurden 20 ccm Saft + 0,2 ccm Toluol unter Zusatz von folgenden Substanzen angesetzt, im übrigen wurde wie in den schon beschriebenen Versuchen verfahren:

A. 1 g Rohrzucker,

B. 1 g Rohrzucker + 1 g Dioxyaceton,

C. 1 g Dioxyaceton.

Die Ergebnisse sind in den Kurven *A*, *B*, *C*, Fig. 1 u. 2, graphisch dargestellt, welche die Kohlensäureentwicklung in der Zeiteinheit angeben. Fig. 1 zeigt ein Frühstadium des Versuches, während in Fig. 2 der Gesamtverlauf der Gärung in kleinerem Maßstabe gezeichnet ist. Aus Fig. 1 ergibt sich, daß bei Zucker (Kurve *A*) ein hoher Anfangs-

---

[1]) Slator, Ber. d. Deutsch. chem. Ges. 45, 43, 1912.

[2]) Das Dioxyaceton wurde aus Glycerin mit Hilfe des Sorbosebakteriums hergestellt. Eine Reinkultur des letzteren war den Autoren durch die Freundlichkeit von Prof. G. Bertrand überlassen worden, die ihm hierdurch ihren besten Dank dafür aussprechen.

wert, 31,6 ccm in 5 Minuten, bedingt durch die Anwesenheit freien Phosphats im Saft, bald erreicht wird. Sobald das Phosphorsalz in

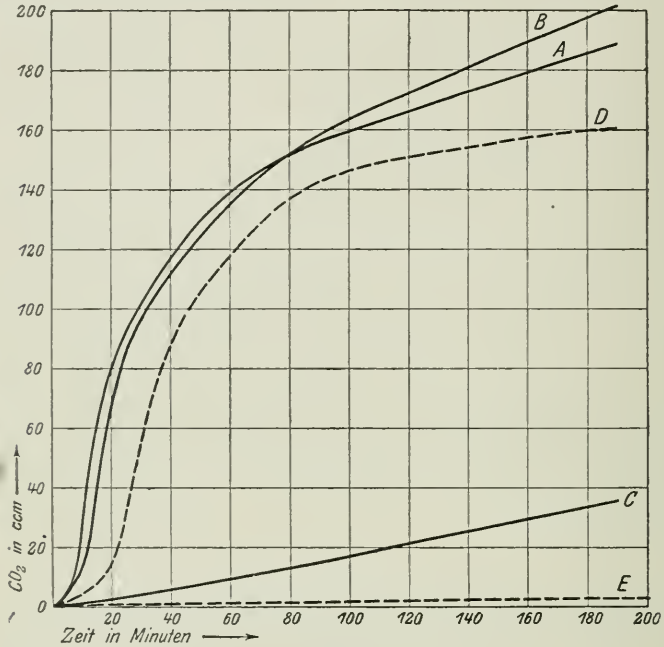


Fig. 1.

Hexosephosphat umgewandelt ist, sinkt er jäh ab, bis er nach 30 Minuten auf 7,2 ccm per 5 Minuten gelangt. Diese Menge bleibt kurze Zeit konstant, was sich bildlich in der Kurvenform zwischen 25 und 55 Minuten ausprägt. Dann nimmt die Menge, mit dem Vorrat des Rohrzuckers Schritt haltend, langsam ab, bis ein Wert von ungefähr 1,7 ccm per 5 Minuten erreicht wird, der von der aus Hexosephosphat entwickelten Zuckermenge abhängt. Wenn diese völlig erschöpft ist, hört die Gärung schließlich ganz auf (Fig. 2). In Gegenwart von überschüssigem Zucker beobachtet man nur die Abnahme in der

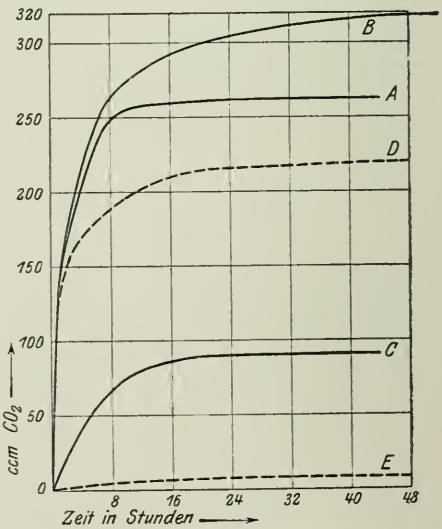


Fig. 2.

durch den Verbrauch des freien Phosphats hervorgerufenen CO_2-Menge, wie dies in den später zu beschreibenden Versuchen über die Wirkung des Konzentrationsgrades bewiesen wird.

Die Dioxyacetongärung *C* verhält sich ganz verschieden. Kein hoher Anfangswert ist zu verzeichnen, vielmehr eine allmählich anwachsende CO_2-Entwicklung (1,2 ccm in 5 Minuten), die langsam abnimmt und zu Ende geht, bevor die theoretisch berechnete Kohlensäuremenge produziert ist.

Kurve *B* zeigt, daß die Gegenwart von Dioxyaceton nicht hemmend auf die Gärung des Rohrzuckers einwirkt. Es treten dieselben Erscheinungen wie in *A* auf, nur mit dem Unterschiede, daß die Gärmengen in langsamerem Tempo abnehmen, und daß Dioxyaceton neben dem Zucker vergoren wird. Das anfängliche Maximum war 28 ccm in 5 Minuten, der nach Erschöpfung des freien Phosphats erreichte konstante Wert 7,3 ccm in 5 Minuten wie bei *A*.

Wenn Dioxyaceton ein Zwischenprodukt bei der Zuckergärung darstellte, dann müßte der Zucker vor der Gärung erst in Dioxyaceton umgewandelt werden und folglich die Gärintensität des Dioxyacetons unter obigen Bedingungen mindestens so groß sein wie die ermittelten Anfangswerte für Rohrzucker.

Daß bei der Gärung von Dioxyaceton noch ein anderer Faktor als bei der Zuckergärung mitspricht, wird durch die weitere wichtige Tatsache bekräftigt, daß, wenn der Saft vor Gebrauch längere Zeit stehen gelassen wird, sein Gärvermögen gegenüber Dioxyaceton schneller als gegenüber Rohrzucker versiegt.

Die punktierten Kurven *D* und *E* in Fig. 1 und 2 stellen die Wirkung auf diese Substanzen desselben Saftes dar, wie er in dem vorhergehenden Versuch zur Verwendung kam, aber nach 3 Tage langem Stehen bei 0°. Die Kurven sind ein Bild der aus folgenden Gemischen entstandenen Gärungen:

D. 20 ccm Saft + 1 g Rohrzucker + 0,2 ccm Toluol.
E. 20 ccm Saft + 1 g Dioxyaceton + 0,2 ccm Toluol.

Sie zeigen, daß der Hefesaft Rohrzucker noch in ähnlicher Weise, aber langsamer als frischer Saft vergärt, während seine Wirkung auf Dioxyaceton nahezu Null ist.

v. Lebedew behauptet, daß Dioxyaceton in Konzentrationen bis zu 5% ebenso schnell wie Zucker vergärt, daß jedoch in höheren Konzentrationen die Gärung eine trägere wird. Seine Erklärung hierfür ist, daß in höheren Konzentrationen das Dioxyaceton eine hemmende Wirkung auf die Zymase ausübt. Zu diesen Schlüssen gelangte er, indem er das prozentisch wirklich

vergorene Substrat verschiedener Konzentrationen von Rohrzucker und Dioxyaceton miteinander verglich. Doch diese Vergleichsmethode ist durchaus irreführend, da bei niedrigem Gehalt die absolute Zuckermenge klein ist und darum vergoren wird, ehe noch das Enzym wirkungslos geworden ist, während bei hohem Gehalt das Enzym schon vor der gänzlichen Vergärung des Zuckers abstirbt. Dasselbe trifft auch für Dioxyaceton zu, nur daß in diesem Falle, da die Gärungsgeschwindigkeit viel geringer als bei Zucker ist, allein bei den niedrigen Konzentrationen das Gesamtdioxyaceton vergoren wird. Unterhalb eines gewissen Verdünnungsgrades hängt deshalb die Gärung von der absoluten Menge der vorhandenen Substanz ab — bei Dioxyaceton liegt er tiefer als bei Zucker. Ein unbedingt richtiger, Fehler vermeidender Vergleich kann nur zwischen den Spannungsgeschwindigkeiten in jedem einzelnen Falle gezogen werden. Ein solcher ist aus den Zahlen, die Lebedew angibt, ausgeschlossen. Auch muß in Betracht gezogen werden, daß ein Teil des Zuckers in Hexosephosphat umgewandelt, weiterhin gespalten und mit geringerer Geschwindigkeit als Zucker vergoren wird, so daß bei kleinen Zuckermengen der wirkliche zu berücksichtigende Gärungswert nur kurze Zeit andauert.

Die Wirkung der erhöhten Konzentration auf die Gärmenge erläutert der folgende Versuch. Die Resultate sind in der folgenden Fig. 3 (S. 472) zur Anschauung gebracht.

Die Kurven stellen den Verlauf der Gärung von 20 ccm Macerationssaft (Schroder) dar, der mit Toluol und wässerigen Lösungen, die im folgenden aufgezählten Substanzen enthaltend, von jedesmal 28 ccm Volumen versetzt wurde.

A: 2 g Fructose,
B: 0,5 g Fructose,
C: 0,25 g Fructose,
D: 2 g Dioxyaceton,
E: 0,5 g Dioxyaceton,
F: 0,25 g Dioxyaceton.

Für Fructose ist, wie ersichtlich, der durch Phosphatgegenwart bedingte hohe Anfangswert in allen drei Fällen der gleiche. Bei *C* wird der ungebundene Zucker bald aufgebraucht, wonach die CO_2-Menge abnimmt, denn das gebildete Hexosephosphat ist nunmehr die einzige Zuckerquelle. Bei *B* ändert sich der Wert zweimal infolge der Erschöpfung zuerst von Phosphat, dann von Zucker. Bei *A* dagegen verringert

sich allmählich nach dem Sinken aus Mangel an ungebundenem Phosphat die CO_2-Menge parallel mit der ersterbenden Enzymtätigkeit des Hefesaftes. Bei Dioxyaceton ist der Anfangswert in allen drei Fällen derselbe und bleibt es die ersten 4 Stunden lang, bis der Vorrat des vergärenden Materials bei den niedrigeren Konzentrationen ausgeht, während bei *D* die Gärung durch den Schwund des fermentierenden Komplexes zum Abschluß gebracht wird.

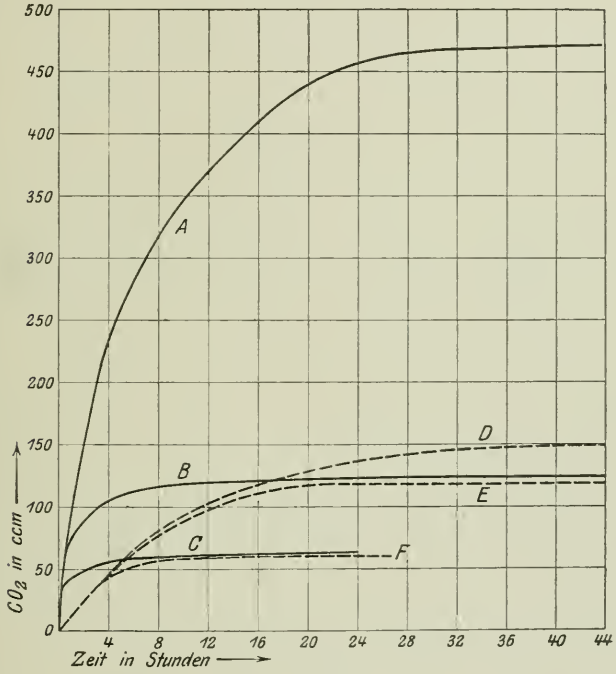


Fig. 3.

Es ist damit aber nicht der Beweis erbracht, daß Dioxyaceton eine größere Hemmung auf die Zymase als Zucker ausübt. Dies würde sich durch eine vorschreitende Abnahme im Anfangswert bei erhöhter Konzentration offenbaren. Wie vorher ist die CO_2-Menge bei Dioxyaceton viel geringer als bei Zucker, und wieder fehlt die beweisende Anfangsbeschleunigung, die auf Phosphatanwesenheit beruht. Es ist zu beachten, daß bei den niedrigen Konzentrationen die Gesamtgärmengen der Fructose trotz so verschiedener Anfangswerte dieselben wie bei Dioxyaceton sind, — das gesamte Substrat wird in beiden Fällen fast gänzlich vergoren.

Ähnliche Resultate erhielten wir auch in einem anderen Versuch, der zahlenmäßig in Tabelle V wiedergegeben ist, mit Macerationssaft aus einem anderen Schroderschen Hefepräparat. Jedesmal wurden 20 ccm Saft benützt, das Gesamtvolumen der Mischung betrug 28 ccm.

Tabelle V.

Zeit	ccm entwickelte CO_2 aus									
	g Dioxyaceton					g Glucose				
Std.	0,25	0,5	1	2	4	0,25	0,5	1	2	4
0,5	2,6	2,7	2,8	3,0	3,4	14,5	18,1	15,3	17,3	15,1
1	5,4	5,2	5,6	6,3	6,5	40,7	50,8	56,7	56,8	50,2
2	9,0	9,9	10,3	10,2	10,4	45,5	79,8	81,2	81,5	75,0
3	11,3	14,0	14,2	14,3	14,5	48,5	86,0	94,0	95,0	89,5
4	12,8	16,8	17,3	17,3	18,1	50,0	90,3	102,0	101,0	97,0
24	19,0	24,8	28,5	28,4	30,0	55,8	102,5	144,2	152,6	169,5
48	20,5	25,8	30,0	30,3	31,0	56,5	104,0	145,0	155,0	175,0

Aus obiger Tabelle geht die sehr wenig intensive Gärtätigkeit dieses Saftes hervor; nur in den Fällen der beiden niedrigsten Konzentrationsgrade der Glucose kam es nahezu zu einer vollständigen Vergärung, in keinem Falle bei Dioxyaceton. Die Tabelle zeigt ferner, daß nicht nur keine Hemmung durch erhöhte Konzentrierung des Dioxyacetons, sondern geradezu ein schwaches Anwachsen in der Gärgeschwindigkeit, wie auch in der gesamten entwickelten Kohlensäuremenge verursacht wurde.

Mehrere Versuche sind mit aus englischer obergäriger Hefe hergestellten Macerationssäften ausgeführt worden. Doch haben wir bisher keine Säfte erhalten, welche, obgleich sie Glucose prompt vergären, Dioxyaceton gegenüber aktiv sind.

Zwei solche Versuche mit zwei verschiedenen Hefeproben sind in Tabelle VI veranschaulicht, die Zahlen beziehen sich auf 20 ccm Saft und 0,2 ccm Toluol für jeden Versuch, bei I. wurde 0,5 g, bei II. 1 g der Substanzen zugesetzt.

Tabelle VI.

	ccm CO_2 in 24 Std.	
	I.	II.
Allein	5,5	6,4
Dioxyaceton . . .	7,6	8,5
Glucose	101,7	239,0

Dieses Resultat liefert neues Beweismaterial dafür, daß bei der Dioxyacetongärung andere Faktoren als bei der Zuckergärung eine Rolle spielen.

Bei dieser Gelegenheit sei darauf hingewiesen, daß die durch Macerierung englischer obergäriger Hefe bereiteten Hefesäfte bei weitem nicht so wirksam wie die aus Münchener Hefe stammenden sind. Ein analoger Unterschied ist auch zwischen den Preßsäften aus englischen Hefen und den von Buchner benutzten bemerkbar und ebenso zwischen den Präparaten aus München und Paris, wie v. Lebedew konstatiert[1]).

Versuche mit Hefepreßsaft.

Eine Anzahl Versuche sind über die Dioxyacetongärung mit Hefesaft ausgeführt worden, der durch Zerreiben von Hefe mit Sand und Kieselgur nach der Buchnerschen Methode dargestellt worden war. Die Beobachtung von Buchner und Meisenheimer, daß Hefepreßsaft allein diese Substanz überhaupt nicht oder nur sehr langsam vergärt, ist von uns bestätigt worden.

Es wurden auch Untersuchungen mit Preßsaft angestellt, dem man Kochsaft, im Vakuum bei 39° zur Hälfte seines Volumens reduziert, zugesetzt hatte. Die Konzentrierung des ungekochten wirksamen Saftes, wie sie von den deutschen Forschern mit Erfolg ausgeführt wird, gelingt mit den Hefesäften englischer Herkunft nicht, da sie während des Prozesses ihre Aktivität einbüßen. Wir benutzten deshalb den unkonzentrierten Preßsaft.

Die Resultate mehrerer Versuche sind in Tabelle VII mitgeteilt, aus der man entnehmen kann, daß nur in Versuch Nr. 9 ein Unterschied zwischen der Selbstgärung und derjenigen in Gegenwart von Dioxyaceton zu verzeichnen ist.

In den vorstehenden Versuchen ist der Unterschied, außer in Nr. 9, zwischen der Selbstgärung und der Gärung bei Anwesenheit von Dioxyaceton so unbedeutend, daß wir es nicht für notwendig hielten, die Gärungsgeschwindigkeiten anzugeben. In Versuch Nr. 9 war die Zunahme in der Gesamtgärung mit Dioxyaceton ebenso deutlich wie mit Glucose, aber der Verlauf der beiden Prozesse gestaltete sich ganz verschieden, wie ein Blick auf Tabelle VIII lehrt.

[1]) Ann. Inst. Pasteur 26, 8, 1912.

Tabelle VII.

Nr.	Saft ccm	Konz. gekocht. Saft ccm	Glucose g	Dioxy-aceton g	CO_2 ccm	Zeit Std.
1	25	—	—	—	126,1	20
	25	—	—	0,5	135,4	20
	25	—	0,5	—	203,9	20
2	20	—	0,5	—	169,3	18
	20	—	0,5	0,5	168,1	18
3	25	—	1,0	—	98,5	3
	25	—	1,0	0,5	95,5	3
4	20	20	—	—	101,5	45
	20	20	—	0,5	112,3	45
5	20	20	—	—	168,1	46
	20	20	—	0,5	173,3	46
6	20	20	—	—	145,0	48
	20	20	—	0,5	147,1	48
	20	20	—	2,0	142,7	48
7	20	20	—	—	138,0	48
	20	20	—	1,0	131,0	48
8	20	20	—	—	133,2	48
	20	20	—	1,0	129,6	48
	20	20	—	4,0	113,4	48
	20	40	—	—	183,2	48
	20	40	—	1,0	184,2	48
	20	40	—	4,0	174,8	48
9	25	25	—	—	397,8	68
	25	25	—	0,5	619,4	68
	25	25	0,5	—	632,6	68

Tabelle VIII.

Zeit		Menge CO_2 im vorhergehenden Zeitraum			Gesamtkohlensäure		
Std.	Min.	Selbst-gärung ccm	Glucose ccm	Dioxy-aceton ccm	Selbst-gärung ccm	Glucose ccm	Dioxy-aceton ccm
0	5	3,8	38,7	3,7	3,8	38,7	3,7
0	10	3,9	35,9	4,2	7,7	74,6	7,9
0	15	3,1	9.3	3,4	10,8	83,9	11,3
0	20	3,0	4,6	3,6	13,8	88,5	14,9
0	25	2,9	3,8	3,7	16,7	92,3	18,6
0	30	2,8	4,0	2,7	19,5	96,3	21,3
3	40	55,2	72,2	63,2	74,7	168,5	84,5
17	25	190,0	217,3	249,4	264,7	385,8	333,9
25	0	64,4	92,6	103,1	329,1	478,4	437,0
41	0	51,0	118,8	121,3	380,1	597,2	558,3
53	0	7,9	24,0	44,3	388,0	621,2	602,6
66	0	8,6	10,4	15,6	396,7	631,6	618,2
68	0	0,7	1,0	1,2	397,4	632,6	619,4

Diese Zahlen zeigen den Unterschied im Verlauf der beiden Gärungen; die Verhältnisse stimmen genau mit denen bei Macerationssaft überein, nur daß in diesem Falle das Gemisch außerdem noch eine erhebliche Selbstgärung ergab.

Versuche mit Zymin.

Zu diesen Versuchen wurde Schroders Zymin benutzt und in einer Koenzymlösung suspendiert, die durch Konzentrierung eines gekochten wässerigen Zyminextraktes hergestellt worden war. Dieser konzentrierte Extrakt enthielt ein aktives Koenzym, dessen Existenz dadurch bewiesen wurde, daß 2 g vorher durch Auswaschen mit Wasser wirkungslos gemachtes Zymin nach Zusatz von Glucose und 10 ccm Extrakt 293 ccm CO_2 entwickelten.

Zwei Versuche mit je 2 g Zymin und 20 ccm Koenzymlösung sind in Tabelle IX zahlenmäßig niedergelegt. In beiden Fällen wurde fast keine Dioxyacetongärung in 48 Stunden beobachtet.

Tabelle IX.

	CO_2 ccm
1. Selbstgärung	90,8
„ + 0,5 Dioxyaceton .	102,1
2. Selbstgärung	99,7
„ + 0,5 Dioxyaceton .	97,0
„ + 0,1 „ .	103,7

Bildung von Phosphorsäureester während der Dioxyacetongärung.

Der folgende Versuch wurde zur Nachprüfung von Lebedews Behauptung angestellt, ob freies Phosphat während des Gärungsprozesses von Dioxyaceton gebunden wird.

Drei Proben Macerationssaft (Schroder) wurden bei 25° im Brutschrank belassen, bis die Temperatur eine konstante war. Nr. 1 wurde dann gekocht, abfiltriert und das ungebundene Phosphat im Filtrat bestimmt.

Zu gleicher Zeit wurde 1 g Dioxyaceton zu 2 und 3 hinzugefügt und die Gärungsmenge beobachtet.

Nach 2 resp. 9 Stunden wurden Nr. 2 und 3 gekocht, filtriert und das freie Phosphorsalz quantitativ ermittelt.

Die in Tabelle X angeführten Resultate beziehen sich auf 20 ccm Saft. Das Phosphat ist in g $Mg_2P_2O_7$ ausgedrückt.

Tabelle X.

Nr.	Entwickelte CO_2 ccm	Phosphat	
		ungebunden	verestert
1	—	0,2389	—
2	27	0,1904	0,0485
3	83,3	0,1896	0,0493

Eine kleine Phosphatmenge ist auf diese Weise gebunden worden, somit ist die Gültigkeit von Lebedews Behauptung experimentell erwiesen.

Mechanismus der Dioxyacetongärung.

Alle vorstehend berichteten Versuche ergeben, daß bei der Gärung von Dioxyaceton ein Faktor miteingreift, der bei derjenigen von Zuckergärung nicht in Erscheinung tritt. Daher kann Dioxyaceton kein Zwischenprodukt bei der alkoholischen Zuckerspaltung sein.

Es drängt sich nun die Frage auf: auf welche Art und Weise vergärt Dioxyaceton? Die darüber bekannten Tatsachen sind folgende:

1. Es vergärt in viel langsamerem Tempo als Zucker.

2. Dabei wird keine typische Phosphatbeschleunigung wie bei Hexose beobachtet.

3. Die Gärprodukte sind nach Buchner und Meisenheimer Kohlensäure und Alkohol.

4. Während der Gärung wird etwas freies Phosphat in eine organische Verbindung übergeführt, die, wie v. Lebedew gezeigt hat, mit dem aus Hexose produzierten Hexosephosphat identisch ist.

Eine mögliche Lösung der Frage könnte nun sein, daß Dioxyaceton langsam in Zucker umgewandelt und als solcher vergoren wird, und diese Eventualität steht durchaus mit allen oben angeführten Tatsachen im Einklange.

Zusammenfassung.

1. Bei Zusatz von Phosphat zu einem Gemisch, bestehend aus Macerationssaft und Zucker, geht mit der schnell sich ent-

wickelnden, dem zugefügten Phosphat entsprechende Kohlensäuremenge eine äquivalente Hexosephosphatbildung einher. Die CO_2 stammt nicht aus der Vergärung von vorher gebildetem Hexosephosphat, wie v. Lebedew behauptet hat. Die beobachteten Phänomene sind also genau dieselben wie bei Zymin und Preßsaft.

2. Die durch Hefepreß- oder Macerationssaft bedingte Gärungsgeschwindigkeit von Dioxyaceton ist geringer als die bei den Zuckerarten erzielte, obgleich Zugabe von Dioxyaceton zu einer gärenden Mischung dieser Säfte mit Zucker die Gärung nicht im ungünstigen Sinne beeinflußt. Dioxyaceton kann deshalb kein Zwischenprodukt der Zuckergärung sein.

Contributions to the Study of Pathogenic Amœbæ from Bombay.

By

W. Glen Liston, Major I.M.S.,

and

C. H. Martin, B.A.,
Demonstrator of Zoology, Glasgow University.

With Plates 16–18.

Part I.—An Examination of Some Cultures of Amœbæ isolated from Dysenteric Lesions and Other Sources. By W. Glen Liston.

Anyone who has attempted to study the literature on amœbic dysentery and liver abscess must have been struck by the confusion existing at present as to the exact differentiation and life-history of the amœbæ, which have been reported as the causative agents of this disease. This confusion can be accounted for, in large measure, by the inadequate and incomplete study of the morphology and development of the amœbæ which have been supposed to be the causative agents.

Schaudinn (**3**) first warned us that all amœbæ to be found in the human intestine were not of the same species. Using the method of building up a life-history from the study of the changes which are observed in a series of individuals at different stages of development—a method which, in his hands, had yielded in many instances splendid results—Schaudinn showed that there were at least two species of amœbæ to be found in the human intestine: the one non-pathogenic, which he named Entamœba coli, the other

pathogenic, which he named Entamœba histolytica. A number of other workers using his methods have discovered still other species, viz. E. tetragena, E. minuta, etc. More recently Musgrave and Clegg (1) in the Philippine Islands, and Noc (2) in French Cochin China, have stated that they have isolated by culture from liver-abscesses and dysenteric stools a somewhat polymorphic amœba, which they regard as the causative agent of liver-abscess and dysentery in their respective countries. They have also found the same species in a number of other situations—in drinking water, on vegetables, and in the intestines of healthy men and animals. In fact, they seem to think that all amœbæ which can be cultivated on their agar medium, and which form cysts varying in diameter between 7 and 16μ, belong to one species,[1] a species which can at one time pass a harmless existence outside the body, at another time, when conditions are favourable, invade the tissues, and give rise to the grave lesions associated with dysentery.

The present paper has been written to show that at least two very distinct species of amœbæ have been found in cultures obtained after the manner of Musgrave and Clegg. I am, however, not yet in a position to state whether both or either of these species are really the causative agents of dysentery and liver-abscess. I am indebted to Captain Wells, of the Indian Medical Service, who has for some time been

[1] While Musgrave and Clegg in their monograph on "Amœbas" (Bureau of Govt. Laboratories, No. 18, p. 77), state that "The cultivation of pure species of amœbas has offered strong evidence of the plurality of the species of these protozoa, and this plurality apparently extends to those which produce infection in man." in a later publication Woolley and Musgrave, in a paper published in the same series, No. 32, June, 1905, write: "It may be well to state at the outset that we can see no valid reason for departing from the nomenclature of Lösch. He described a pathogenic amœba and called it Amœba coli. Why this term should be applied to a suppositious non-pathogenic organism it is difficult to say. We shall, in referring to the cause of intestinal amœbiasis, use the name introduced by Lösch." The measurements of the cysts are those given by Noc.

engaged in the study of dysentery in India, for the cultures I have used. These cultures of amœbæ I understand were obtained in Bombay from the following sources: (A) a liver-abscess; (B) a dysenteric stool; (C) Bombay City tap-water; (D) the stool of a healthy monkey.

A period of leave to England presented me with a favourable opportunity for studying these cultures under the control and guidance of a skilled protozoologist. I have to thank Dr. Martin, the Director of the Lister Institute, and Professor Minchin, of the London University, for allowing me to work in the laboratory at the Lister Institute, and the latter especially for drawing up for me a scheme of research in connection with the cultures I had obtained, and for instructing me in the best methods for studying the amœbæ.

Professor Minchin suggested that I should endeavour to ascertain whether the amœbæ from these different sources were one and the same species, by studying their morphology and development in cultures, and, by using the physiological test, attempt to produce dysenteric lesions in animals. He further suggested that I might find out whether these parasitic amœbæ absorb food osmotically, like a trypanosome, or must devour things like the ordinary free-living amœbæ. I was to endeavour, if possible, to get pure cultures free from bacteria.

A preliminary examination of the cultures, which had been planted more than a month previously, showed that in each case all the amœbæ were encysted, and that in all they were associated with a variety of bacteria. The size of the cysts in the different cultures varied considerably, but it was observed that on the whole the cysts in the culture obtained from a dysentery-stool were smaller than those found in the cultures obtained from Bombay tap-water and from the monkey. The cysts in the liver-abscess culture varied more considerably than the other cultures; many very small cysts, often grouped together, were mixed with cysts as large as those found in the water or the monkey culture. It appeared, then, that in the case of the liver-abscess culture two types of cysts were present. Measurements of the cysts showed

that while the smaller type varied in diameter between 6 and 8 μ, the larger types varied in diameter between 12 and 15 μ. To determine whether the amœbæ which developed from these cysts remained true to type, a single cyst was isolated under a lower power of the microscope on the point of a brush or needle which had been dipped in gum, and the cyst was then transferred to fresh culture-medium. It was found that the amœbæ always remained true to type, that is to say, large cysts were always formed by amœbæ which had developed from a single large cyst, while small cysts were always the product of small cysts. Moreover, the amœbæ from the large cysts were considerably larger than the amœbæ from the smaller cysts. There thus appeared at first sight to be two species of amœbæ present in the liver-abscess culture. This supposition was further substantiated by the fact that the other two types differed in respect to (A) their methods of multiplication; (B) their behaviour in saline solution; and (C) in other minor points. These points of difference will now be discussed.

(A) Methods of Multiplication on Culture-medium.

The large amœba was observed to multiply by endogenous budding as well as by division into two individuals. On one occasion a living amœba in a pure culture derived from large cysts was watched for some hours. During this period three or four buds were formed within the body of the amœba and liberated from it. This method of multiplication was more easily followed in fixed and stained specimens. Budding was observed both in old and in young cultures. In one case, an amœba, which from its size appeared only lately to have emerged from its cyst, already contained a bud almost as large as itself (Pl. 17, fig. 21), while an older and larger amœba showed as many as six buds in various stages of development (Pl. 16, fig. 4). It will be observed that the method of budding here demonstrated (Pl. 16, figs. 1–5, Pl. 17, figs. 16–20) differs materially from that described

and figured by Noc (2), who describes the buds he observed as very small, each bud containing a single minute fragment of chromatin. So numerous were the buds within the body of the parent amœba that he says the term "merozoite" could be aptly applied to the bred. In my experience great difficulty was found in differentiating between the bodies of bacteria ingested by an amœba and lying within its cytoplasm and small fragments of chromatin or chromidia. It must be remembered than in cultures the amœbæ are always present with vast numbers of bacteria. Although a number of stains were used none was found which satisfactorily distinguished bacteria from chromidia. It was therefore difficult to trace the origin of the buds. The buds became recognisable within the cytoplasm of the parent amœba, when a larger mass of chromatic material was assembled than could be reasonably explained on the supposition that it was formed from ingested bacteria. Around these collections of chromatic material the cytoplasm of the amœba becomes differentiated from the rest of the cytoplasm of the body. As this differentiation becomes more and more marked the enclosed chromatic material more and more closely resembled the structure of a typical nucleus, till finally, within the body of the amœba, a completely differentiated amœbula is developed. At this stage the amœbula escapes from the parent amœba, and can then live a free existence, grow and develop into a full-sized adult amœba.

Division of this large amœba from the liver-abscess culture into two individuals was also observed in the living condition. On one occasion a single amœba was watched continuously for two hours and fifty-four minutes, and a detailed record was kept of the various events that occurred during this period. The culture in which this amœba was observed had been planted five and three-quarter hours before the observations commenced. A number of amœbæ had already escaped from their cysts while still others had not yet emerged from their cysts. The amœba which was selected for observation was a very active one. In progressing over

the culture-medium it made its way by extruding two distinct types of pseudopodia, viz. (1) coarse lobose pseudopodia, consisting almost entirely of ectoplasm, were extruded for the most part in the direction of movement, and (2) fine, needle-like, short, pointed pseudopodia extended out from the lobose pseudopodia as well as from other parts of the body. A nucleus and contractile vacuole were plainly visible. The vacuole contracted rhythmically and fairly regularly almost every two minutes. The amœba moved over the medium at about the rate of 160 μ in thirteen minutes. As it progressed it encountered in its path little groups of cocci which were growing with it on the culture-medium. During one hour and thirty-eight minutes before the amœba divided into two individuals, one hundred and eight cocci were ingested by the amœba under observation. The cocci were taken into the protoplasm of the amœba in groups of two or four. When larger groups of cocci were encountered the amœba broke them up into smaller groups and then ingested them. Four minutes after ingestion the cocci were seen to be contained within a vacuole. The vacuoles containing the cocci moved about through different parts of the protoplasm, and as digestion proceeded they at first became larger, then, becoming smaller, they slowly disappeared. (On a neutral-red medium it was easy to demonstrate that the contents of the vacuoles had an acid reaction.) The cocci within the vacuoles for a time remained visible, but gradually, in the course of fifteen minutes, melted from view. After watching the amœba for one hour and thirty-eight minutes, it gradually became more and more sluggish and the contractile vacuole contracted less and less frequently. These changes occurred immediately after the amœba had engulfed piecemeal a group of thirty cocci, the amœba appearing to be satiated with this big meal. The amœba had meanwhile assumed a rounded form, withdrawing all lobose pseudopodia, but still the fine needle-like pseudopodia projected from its surface. The movements of the protoplasm, which up to this time had been active, gradually ceased altogether, and

the protoplasm became more and more granular. The nucleus, which up to this point had been clearly visible, could now no longer be distinctly defined. No movement of any sort was noticed for a few minutes. Some ten minutes after the amœba had engulfed the last cocci, and a few minutes after all movement had ceased, the amœba slowly changed from a rounded to a more oval form, becoming oblong, then elongated. Two small indentures next appeared in the protoplasm on either side, about the middle of the body. These indentures deepened, till, meeting, two amœbæ were formed. Division was complete in three minutes after the first appearance of the indentures mentioned above, and about thirteen minutes after the last cocci had been taken up as food. No trace of a nucleus could be made out in either of the two new amœbæ immediately after division. The newly formed amœbæ soon moved away from one another, and in three minutes one of them had approached and engulfed a group of nine cocci. A nucleus now gradually appeared in each amœba, but not till five minutes after division had been completed could it be clearly defined, meanwhile, as has been remarked above, the amœbæ moved about and even fed. Each amœba by this time had a contractile vacuole which rhythmically contracted about once every two minutes. The young amœbæ were watched for nearly an hour longer; no fresh points of interest, however, were noticed. The observations were abruptly terminated by an accidental knock to the Petri dish, which caused the amœbæ under observation to be moved out of the field of vision, and made it impossible to recognise with certainty those which had been watched from other amœbæ in the culture. I have not been able successfully to follow in a series of stained specimens the various changes described above seen in the living amœbæ. (Since writing this, with the assistance of Mr. C. H. Martin, divisional forms have been studied in stained specimens. The division is indirect or by mitosis.)

While thus the large amœba from the liver-abscess culture

showed two methods of multiplication, the small amœba, cultivated from the small cysts of the same culture, was never seen to give off buds. Actual division in the living state was not observed in the case of this amœba, but in stained preparations dividing forms were fairly easily found. Division in this case is direct or amitotic. Three such forms have been sketched from a single slide, while in other slides other stages of division have been observed.

(B) The Behaviour of the Amœba in Saline Solution.

One of the most striking differences between the two species of amœbæ found in the liver-abscess culture was brought into evidence when dilute saline solution 5 per cent. was added to an actively developing culture, time being allowed for the amœbæ to recover from the shock produced by the addition of the liquid. To demonstrate this difference between the two species of amœbæ the best results were obtained by placing a drop of the mixture of the amœbæ and saline solution on a slide and allowing it to remain for a few minutes in a warm moist chamber before fixing in Flemming's solution or sublimate-acetic mixture. The drop containing the amœbæ must not be covered with a cover-glass, but should be spread out in a thin layer and left freely exposed in a moist chamber. Adopting this method of preparing and fixing specimens, it was found that the large amœba always assumed a more or less rounded form, with lobose and numerous fine needle-like pseudopodia projecting from the surface. The small amœba on the contrary almost always assumed an elongated worm-like or gregarine shape, throwing out one or two long lobose pseudopodia either directly in front or often from the side so that L-shapes and Y-shapes, as well as long I-forms, were developed.

That Noc was dealing with both these types of amœbæ is evident, not only from the measurements of the cysts he worked with, but also from the drawings on his plate X. The drawings show both forms, the majority representing the

large amœba type, while his fig. 20 is a typical specimen of the small amœba described above.

(c) Minor Points of Difference.

Other less important differences were noted between the two types of amœba found in the liver-abscess culture. Thus, for example, the nucleus of the large amœba in the living state was more clearly seen than that of the small amœba. The ectoplasm, too, was more clearly differentiated from the endoplasm in the case of the large amœba than in the small amœba. Then, again, a yellow pigment-producing coccus, which was a peculiarly favourable organism to grow with the large amœba, appeared to be quite unsuitable for the smaller amœba.

From what has been said above, it is evident that two very distinct species of amœba were present in this liver-abscess culture, and although a somewhat limited amount of attention has been given to the cultures derived from other sources, it is possible that the cultures from the Bombay City water and from the monkey, which contained an amœba very closely resembling the large amœba from the liver-abscess culture, nevertheless differed from it. The small amœba from the liver-abscess culture very closely resembled that found in the culture from a dysenteric stool.

An attempt was made further to differentiate between the two species of amœbæ found in the culture from liver-abscess by carrying out experiments on animals. For the purpose of these experiments young kittens were selected. The two species of amœbæ were isolated in pure mixed culture, and, when the amœbæ had encysted, an emulsion of each species was made in saline solution. Four kittens, whose fæces in the first instances had been examined microscopically and by culture for the presence of amœbæ with negative results, were experimented upon as follows: Two were fed by means of a small stomach-tube, the one with an emulsion of large amœba-cysts, the other with an emulsion

of small amœba-cysts. The two other kittens were injected per rectum, the one with an emulsion of large amœba-cysts, the other with an emulsion of small amœba-cysts. On the following day, as well as on the third day after treatment, the stools were examined both microscopically and by cultures for the presence of living amœbæ, but none were found. The kittens were healthy a fortnight later. These physiological tests were, then, unsuccessful in demonstrating any pathogenic properties in either species of amœba found in the liver-abscess culture, and they also failed to distinguish the one species from the other. Too much stress, however, cannot be placed on the failure of these experiments, not only because of their small number, but especially when the complicated conditions which are associated with the living together of bacteria and amœbæ are kept in mind, and to which some reference will be made later. Nevertheless, the negative results of these experiments are in conformity with the more numerous ones carried out by Noc and reported by him (**2**). By way of contrast, I found it interesting to study sections of the large intestine of a cat which had been infected with dysentery by the rectal injection of the stools of a patient suffering from dysentery in the Straits Settlements. This material was kindly supplied to me by Dr. Ledingham. Sections of this tissue showed well-marked dysenteric lesions associated with the presence of amœbæ, the amœbæ being found together with bacteria, not only in the mucosa, but penetrating into the submucosa in the neighbourhood of the ulcerations. It is interesting to compare the morphological appearance of these amœbæ with those found in the cultures. In the first place, the amœbæ in the sections of the cat's intestine were considerably larger than even the larger type found in my cultures. The nuclear structure of these amœbæ, too, differed remarkably from that of the amœbæ of the cultures. Thus, while the chromatic substance of the nucleus of the cultural amœbæ was abundant and differentiated into a large central portion and a thin peripheral layer, the chromatic substance of the

nucleus of the amœbæ found in the sections was very scanty, and confined for the most part to the periphery of the nucleus. These observations are in accord with those of Noc, who, when writing of the amœbæ which he isolated in culture and comparing them with Schaudinn's E. histolytica, and living forms isolated from the walls of a liver-abscess, points out that the cultural amœbæ were always smaller and contained more chromatin in the nucleus than those found in the tissues of a liver-abscess or of a dysenteric ulcer. How far these differences can be accounted for by differences in the methods of fixation and by the nature of the medium in which the amœbæ have respectively developed it is difficult to say, yet it is evident that the amœbæ which grow in cultures differ markedly in morphological characters from those found in sections of diseased intestine. Further study with fresh pathological material seems to be necessary before any definite conclusion can be arrived at as to whether the amœbæ obtained in cultures are the true cause of dysentery.

A few more details of the development of the amœbæ in cultures remain to be mentioned. The following observations concern particularly the large amœbæ of the liver-abscess, which was studied in greater detail than the smaller amœbæ. A single amœba has frequently been watched as it became encysted. Coming to rest the amœba gradually shrinks and becomes condensed. During this period the contractile vacuole beats more and more slowly; finally, having attained often an unusually large size, it very slowly shrinks and disappears. Other vacuoles in the protoplasm also disappear. Then a cyst-wall develops around the amœba. A single nucleus is alone visible within the cyst as long as it remains transparent and its contents can be stained.

When cysts formed in this way are planted on fresh medium the first change that is noticed is a gradual swelling of the cyst and the development of an increasingly large vacuole within it. The cyst-wall soon ruptures at some indefinite point and a single amœba slowly flows out through the breach.

It is apparent that when conditions are unsuitable for the growth of the amœba, a single individual by condensation of its protoplasm and the development of a cyst-wall becomes encysted, and, when the conditions are favourable, a single amœba escapes from this cyst. No evidence of conjugation was observed before encystment and no multiplication or division of the nucleus noticed, either immediately before or after encystment. Encystation appeared to be a purely protective measure. Outside the body the amœba was able to feed and to multiply under favourable circumstances, but it encysted when circumstances were unfavourable for development.

I will pass on now to describe some experiments which were made with a view to finding out whether the large amœba of the liver-abscess culture could absorb food osmotically and live and multiply on the agar medium in the absence of bacteria.

Much time was spent in the first instance in attempting to get the amœbæ from the four sources mentioned above to grow with a single species of bacteria. This was comparatively easily accomplished with the large amœba from the liver-abscess culture, but with greater difficulty in the case of the other amœbæ. All the "pure mixed" cultures were ultimately obtained with motile organisms except in the case of the large amœba from the liver-abscess. In this case the amœba was cultivated with a variety of motile and non-motile organisms, but particularly with a non-motile, yellow, pigment-producing coccus. With this pure mixed culture a number of experiments were conducted, and these are detailed below.

This amœba with the non-motile coccus multiplied enormously on the agar medium. The young amœbæ moved away from the immediate neighbourhood of the bacteria and ultimately became encysted. Using such a culture it was comparatively easy to separate amœba cysts from bacteria. With these cysts free from bacteria a number of experiments were carried out.

(1) A single cyst, free from bacteria, was isolated and

placed on fresh agar medium. A single amœba twelve hours later was found to have escaped from the cyst and was moving over the medium. Forty-eight hours later a single amœba was alone still actively moving over the culture medium; no multiplication had occurred. On the third day the amœba had become encysted again. In this case a single amœba had emerged from a single cyst and moved about for two days on the culture medium, which was free from any bacterial growth; no multiplication had taken place, but on the third day encystation had again occurred.

(2) A few cysts free from bacteria were planted out on an agar medium which, in the first place, had been smeared on one occasion with fresh sterile mouse's liver, on another occasion with fresh sterile unheated guinea-pig's liver. The amœbæ in due course emerged from their cysts and were observed to feed upon the red blood-cells and broken-up liver-cells of the smeared agar surface. The liver-cells which were not damaged or broken were too large to be engulfed by the amœbæ, which were approximately of the same size as these cells, some amœbæ, however, made attempts to swallow whole liver-cells, spreading themselves around the cells in a thin layer, but never succeeding in completely surrounding the liver-cell. A single amœba on one occasion was watched for two hours and a half while moving about on the agar medium smeared with fresh sterile guinea-pig's liver. During this time this amœba ingested and digested four red blood-cells, and attempted as well to take up a number of liver-cells as large as itself.

While thus the large amœba of the liver-abscess culture was seen to feed upon red blood-cells and broken down liver-cells, very little multiplication of the amœbæ was noted. Generally, without dividing, the amœbæ became encysted again. This was inferred from the fact that the number of cysts found after some days was very little in excess of the number originally placed on the medium. With the object of stimulating multiplication of the amœbæ the following procedure was adopted:

A number of sterile agar plates were smeared with fresh sterile guinea-pig's liver. These were placed in the incubator for two days to make sure that no bacteria had been planted on the surface of the medium during the preparation of the plates. Then to plate (A) a drop or two of an emulsion of the yellow coccus in saline solution, which had been boiled, was added at the same time as some bacteria-free cysts were planted on the liver-smeared culture. To (B) a drop or two of sterile saline solution was added as well as a few bacteria-free cysts. To (C) a drop or two of very weak sterile sodium carbonate solution with bacteria-free cysts was added. To (D) a few living yellow cocci in salt-solution were added with bacteria-free amœba-cysts.

The plates were then placed in the incubator and on the following day were examined. In each case living and moving amœbæ were observed. The plates were kept under observation for some days longer, and it was noted that while very little multiplication of the amœbæ had occurred in cultures A, B, and C in D, in the neighbourhood of the colonies of cocci, the amœbæ had multiplied enormously.

So far as it is possible to judge from a single experiment like the above, it would appear that multiplication of the amœbæ only occurs in the presence of some substance which is apparently connected with the life of the bacteria, a substance which is destroyed by boiling. In this connection the following casual observations are worthy of record: On a number of occasions it was noticed that if the number of cocci planted with the amœba-cysts was considerably in excess of the number of cysts, and particularly if the medium in which the culture was made favoured the development of the cocci, the amœbæ failed to develop, while the cocci flourished. On two or three occasions, when apparently a culture of amœbæ was growing well and multiplying in the presence of a colony of the yellow coccus, a stage was reached when the bacteria appeared to have produced some secretion which, diffusing outwards from the colony, caused those amœbæ which were in the immediate neighbourhood of the

colony, and which had not become encysted, to be dissolved, the amœbæ appearing to break up, melt, and disappear. On one occasion this condition was brought about by stirring up and spreading a little of the yellow coccus colony which was growing in the midst of a luxuriant culture of amœbæ.

These observations lead me to believe that a very delicate balance is maintained in a successful culture of amœbæ and bacteria. While on the one hand the amœbæ eat up and digest the bacteria, the bacteria, on the other hand, seem to produce some substance or substances which at one time stimulates the amœbæ to multiply, at another time actually brings about their destruction.

From what has been said above it is evident that I have not succeeded in showing that this amœba can absorb its food osmotically or live successfully without living bacteria; but I think certain lines for future research have been opened up which may ultimately explain the necessary connection which seems at present to exist between bacteria and the development and multiplication of amœbæ outside the body.

No one can be more conscious of the incompleteness of the work detailed above than myself. This paper has been written only because an opportunity may not present itself in the immediate future to continue this inquiry on my return to duty in India. It seems to me important, in the presence of a number of different species of amœbæ which may be found inhabiting the human intestine or in dysenteric lesions, that greater care should be taken in distinguishing one species from another, and specially the pathogenic from harmless commensal amœbæ.

Conclusions.

Two distinct species of amœbæ isolated from a liver-abscess have been cultivated on an agar medium. One at least of these amœbæ in cultures does not multiply in the absence of living bacteria. The same bacteria which, when

alive, stimulate the amœbæ to multiplication, when boiled and eaten by the amœbæ lack this power.

Amœbæ have been seen to feed upon and digest red blood-corpuscles, but in the absence of living bacteria failed to multiply on agar cultures.

LISTER INSTITUTE.

November, 1910.

Part II.—Descriptions of Preparations of Amœbæ from Major Liston's Cultures. By C. H. Martin.

In November of last year, Major Liston was kind enough to hand over to me some preparations and cultures of amœbæ, with a request that I should look through them. The preparations were made from cultures of five different strains of amœbæ, under the circumstances which Major Liston has described in Part I of this paper.

Major Liston has given an account of his observations on three of these forms, and since the live cultures unfortunately dried off in my hands before sub-cultures could be made, it only remains for me to add a few notes on the details of division and budding from the stained films of the larger type of amœba from liver-abscess. I have also, for the sake of completeness, given a short description of the other amœbæ, in the hope that I may be able at some future date to give the results of further work on live cultures.

THE LARGER TYPE OF AMŒBA FROM LIVER-ABSCESS.

As will have been seen already from Major Liston's description, this is a very well-marked form, both from a morphological and a physiological standpoint. In stained preparations the full-grown amœba is characterised by a nucleus in which the mass of the chromatin is condensed into a large round karyosome (Pl. 16, figs. 1–5). In addition to the chromatin of the karyosome, there is a cloud of fine

granules forming a peripheral zone close beneath the nuclear membrane. With Twort's combination of neutral red and Lichtgrün (Pl. 17, figs. 17–20), or when preparations stained with iron-hæmatoxylin are counter-stained with Lichtgrün (figs. 14–16, 21), the peripheral zone is coloured green, a reaction which indicates that achromatic elements predominate in this region, and that a true achromatic membrane is present enveloping the nucleus. The dense karyosome frequently shows a central lighter region in which one or two darker grains, doubtless of the nature of centrioles, can be made out.

The numerous food-particles (chiefly ingested bacteria) which are present in the cytoplasm, and which take up the ordinary nuclear stains very strongly, render the precise study of the chromatin-grains very difficult; but in some well-stained examples there seems to be distinct evidence of passage of chromatin-granules into the cytoplasm, where they may form a more or less irregular chromidial mass surrounding the nucleus or scattered through the cytoplasm, which, consequently, is coloured a more or less deep red with the neutral red combination (Pl. 17, figs. 17–20). It will be necessary to return to this point in connection with the phenomena of budding.

As has been stated above, the large amœbæ in the liver-abscess may reproduce in the cultures in one of two ways: (1) by simple division with karyokinesis; (2) by the formation of endogenous buds.

(1) Division.—The main features of the behaviour of the nucleus in this case make it clear that the process of division is a mitosis, and this is fully confirmed by Major Liston's live observations. Unfortunately I have not been able to obtain evidence as to the method in which the nuclear spindle is formed. In the earliest examples of division that I have been able to recognise the nuclear spindle is already fully formed (Pl. 16, fig. 7), and the chromatin of the nucleus lies in the equatorial plate in the form of a number of rather irregular masses. In the later stages of division (Pl. 16,

figs. 8 and 9) the chromatin-masses have divided and separated slightly, and it is, I think, interesting to note, from a comparison of the early and late stages of division-figures (Pl. 16, fig. 7 and fig. 10), that the separation of the chromatin-masses does not appear to be due in any marked degree to the shortening of the polar threads of the spindle between the centrioles and the chromatin-masses, but rather to a growth and elongation of the separation-spindle between the two daughter-plates. In the still later stage of division shown in Pl. 16, fig. 11, the upper nucleus has practically assumed the resting condition, and would seem to show that the chromatin of the daughter-plate forms the mass of the karyosome, in which the centriole is probably included.

(2) Budding.—The most common form of reproduction for the larger type of amœbæ from liver-abscess is effected by the formation of fairly large buds, as has been described already by Major Liston in the account of his observations on the live amœbæ. There is no evidence on the stained films of this form for the formation of the numerous very small buds described by Noc (2) for a similar amœba obtained by him from liver-abscesses in Cochin-China. In the majority of the forms studied by us only one bud is formed at a time, though there are cases where this number may be exceeded (Pl. 16, fig. 4 and fig. 10).

The nucleus of the amœba takes no direct part in the formation of the bud. There is absolutely no evidence, either from observations on the live amœbæ or from the stained films, for any form of nuclear division connected with the bud-formation; and, on the other hand, it is quite clear from the stained preparations that the cytoplasm of the bud is completely cut off from that of the parent at a stage in which the bud shows no distinct nucleus. A comparison of different stages in the growth of the bud shows that the nucleus of the bud arises from chromidia contained in it when it is first formed, and derived from the chromidia scattered through the cytoplasm of the parent.

When first recognisable, the bud is seen as a small sphere,

about 3 μ in diameter, separated from the surrounding cytoplasm of the parent by a clear space (Pl. 16, fig. 6; Pl. 17, fig. 19); at this stage the substance of the bud shows no difference from that of the parent cytoplasm, except that with Twort's stain it stains a deeper red (Pl. 17, fig. 19), indicating that the chromidia are present in greater quantity, probably as the result of growth and increase of the chromatin-substance in the bud itself after it has been cut off from the parent. The bud, imbedded completely in the maternal cytoplasm, grows until it attains a diameter of 9–10 μ or more. The chromidia contained in the bud condense into irregular strands of chromatin (Pl. 16, figs. 2 and 3; Pl. 17, figs. 17, 18), which gradually become arranged into a nucleus of the type of that possessed by the parent (Pl. 16, fig. 4; Pl. 17, fig. 19). But by no means all the chromidia contained in the bud are used up to form its nucleus; a certain number remain over (Pl. 16, figs. 3, 4, 6; Pl. 17, figs. 18–21), so that the fully formed bud contains chromidia in its cytoplasm in addition to a nucleus. The bud at the time of its escape may show a well-developed nucleus with a karyosome (Pl. 16, fig. 4), but more frequently this change is completed after the young amœba is set free (Pl. 16, fig. 6). The young amœbæ begin to form buds in their turn long before they are full grown (Pl. 17, fig. 21).

The question of the cysts is rather a difficult one, and needs further work on live cultures. In smears from cultures about eight hours old amœbæ may be seen still enclosed in a smooth-walled cyst with the vacuole which Major Liston has described as being developed in the escaping form (Pl. 16, fig. 12). On the other hand, empty rough-walled cysts are also found. The latter may be due to a shrinkage of the cyst after the amœba has escaped, but it must be confessed that they seem rather too large to be explained on this hypothesis (Pl. 16, fig. 13).

The Smaller Type of Amœba from Liver-Abscess and the Dysentery-Amœbæ.

The small type of amœba from liver-abscess (Pl. 18, figs. 22–24), and the dysentery-amœbæ (Pl. 18, figs. 25–29), seem

to agree absolutely in regard to all their essential features. In both these types it seems rather difficult to stain the nucleus satisfactorily, and moreover, there seems to be evidence for a series of remarkable morphological changes in the structure of the nucleus of this form, the significance of which is not at present clear to me (Pl. 18, figs. 22–24). From the observations on the live cultures it is evident that the nuclear division is amitotic, and neither from such observations nor from the stained films is there any evidence whatever in this type for the occurrence of endogenous budding. Until more time has been spent on this form, and fresh material obtained of it, it is impossible to estimate to what extent it may differ from Schaudinn's Entamœba histolytica.

As regards the other two amœbæ, that from the monkey's rectum (Pl. 18, figs. 31, 32) was on the stained films a roughly spherical form, with rather darkly staining cytoplasm, measuring about 15 μ in diameter. The cytoplasm contained numerous vacuoles. In the nucleus the chromatin was chiefly condensed in the large central karyosome and in a distinct peripheral zone of fairly large granules. The amœba cultivated from Bombay tap-water (Pl. 18, fig. 30) measured about 16 by 10 μ; its cytoplasm was clear, and in some cases markedly vacuolated. The nucleus showed the chromatin massed in a central karyosome.

Although I am sure that further work on these forms would lead to interesting and valuable results, yet I feel that there is already evidence amply sufficient to prove Major Liston's main contention that the larger amœba from liver-abscess is quite a distinct type, different from the other small form here described; in fact, when one regards the simple method of reproduction by endogenous buds seen in this form, it may be doubted whether it ought to be included in the amœbæ at all, but having regard to our present ignorance of this form, it may be appropriate to leave it for the present in the group which Schaudinn has pithily described as "ein Sammeltopf der heterogensten Elemente."

Lister Institute,
April 4th, 1911.

References.

1. Musgrave, W. E., and Clegg, M. T.—"Amœbas: their Cultivation and Ætiologic Significance," 'Manila, Dept. of the Interior, Bureau of Government Laboratories, Biol. Lab.,' No. 18, 85 pp., 32 figs. (1904).

2. Noc, F.—"Recherches sur la dysenterie amibienne en Cochin-Chine," 'Ann. Inst. Pasteur,' xxiii, pp. 177–204, pls. x–xiii (1909).

3. Schaudinn, F.—"Untersuchungen über die Fortpflanzung einiger Rhizopoden," 'Arb. k. Gesundheitsamt. Berlin,' xix. pp. 547–576 (1903).

EXPLANATION OF PLATES 16–18,

Illustrating Major W. Glen Liston and C. H. Martin's "Contributions to the Study of Pathogenic Amœbæ from Bombay."

[All the figures are drawn with the camera lucida to a magnification of 2000 linear, using Zeiss objective apochr. 3 mm. homog. imm. N.A. 1·40, compens. ocular 18.]

PLATE 16.

Large type of amœba from liver-abscess. All the preparations are stained with Heidenhain's iron-hæmatoxylin, after fixation with Schaudinn's fluid or sublimate-acetic.

Figs. 1–6.—Stages of endogenous bud-formation.

Figs. 1 and 2.—Early stages; in fig. 2 irregular chromidial strands are seen in the bud.

Fig. 3.—Later stage; the chromidial strands are condensing to form the nucleus of the bud.

Fig. 4.—Amœba showing six endogenous buds, one of which has the nucleus completely formed.

Fig. 5.—Extrusion of a fully-formed bud.

Fig. 6.—Free bud, with nucleus not quite fully-formed.

Figs. 7–11.—Karyokinesis and cell-division.

Fig. 7.—Early stage; the spindle is fully-formed, but the equatorial plate is not split.

Fig. 8.—Splitting of the equatorial plate.

Fig. 9.—The daughter-plates separating.

Fig. 10.—The daughter-plates widely separated; reconstitution of the daughter-nuclei beginning.

Fig. 11.—End of nuclear division; one daughter-nucleus is completely reconstituted, the other shows remains of the spindle. Division of the cell-body beginning.

Fig. 12.—Encysted amœba, about to escape from the cyst.

Fig. 13.—Empty cyst with rough wall.

PLATE 17.

Large type of amœba from liver-abscess. Figs. 14–16 are counter-stained with Lichtgrün-picric after iron-hæmatoxylin; figs. 17–20 are stained with Twort's combination of neutral red and Lichtgrün.

Figs. 14 and 15.—Amœbæ with ingested bacteria; in fig. 14 the karyosome appears to be breaking up.

Figs. 16–21.—Amœbæ showing endogenous bud-formation.

Fig. 16.—Amœba with two buds, one very small, in the earliest stage of formation, the other full-sized, with the chromidia beginning to form the nucleus.

Fig. 17.—Amœba with full-sized bud, which contains only scattered chromidia.

Fig. 18.—Similar stage, the nucleus of the bud beginning to be differentiated.

Fig. 19.—Amœba with three buds, two in a very early stage of formation, the third full-grown, with nucleus completely differentiated.

Fig. 20.—Amœba containing fully-formed bud.

Fig. 21.—Young amœba, not full-grown, containing a bud which is full-sized and has the nucleus in an advanced stage of differentiation.

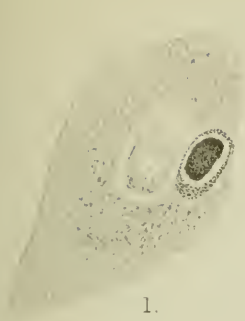
PLATE 18.

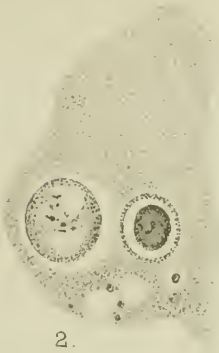
Figs. 22–24.—Small type of amœbæ from the liver-abscess, showing stages of the division of the nucleus. Stained with Delafield's hæmatoxylin.

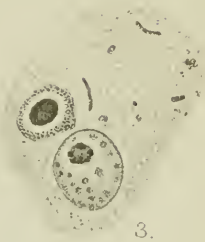
Figs. 25–29.—Amœba from dysenteric stools. Figs. 28 and 29 show early stages of nuclear division. Iron-hæmatoxylin.

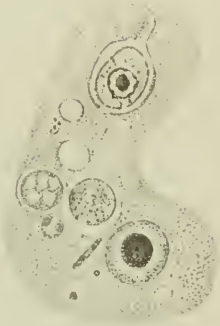
Fig. 30.—Amœba isolated from Bombay tapwater. Iron-hæmatoxylin.

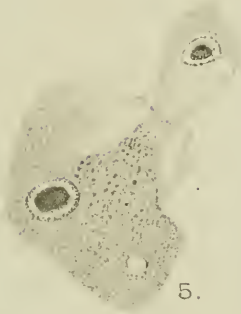
Figs. 31 and 32.—Amœbæ from the rectum of a monkey. In fig. 27 the nucleus has recently divided. Iron-hæmatoxylin.


1.

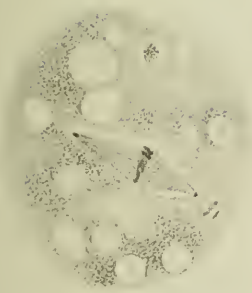

2.

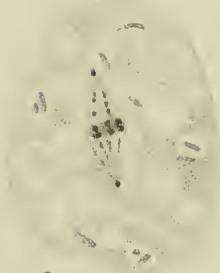

3.

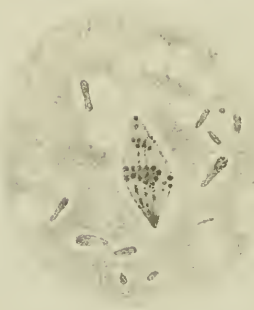

4.

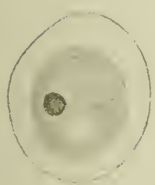

5.


6.

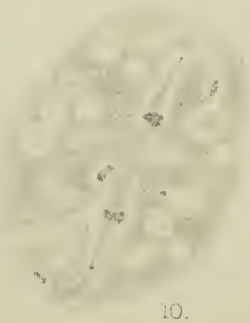

7.


8.


9.


12.


11.


10.

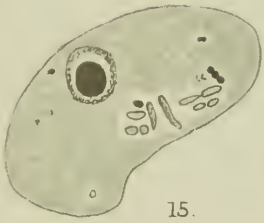

13.

M. Rhodes del.

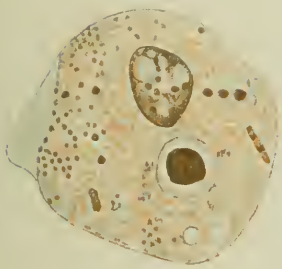
AMŒBÆ FROM LIVER-ABSCESS.

Hath, Ladd London

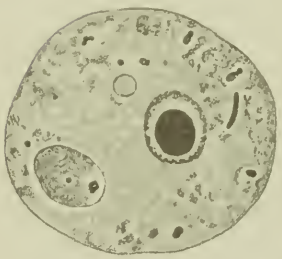
14.



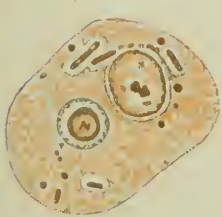
15.



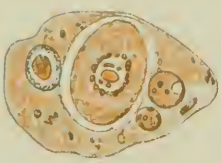
17.



16.

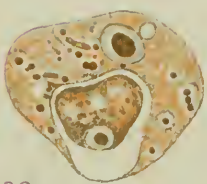


18.



19.

21.



20.

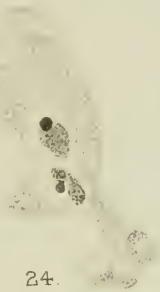
M R del.

Huth, Lith^r London.

AMŒBÆ FROM LIVER-ABSCESS.

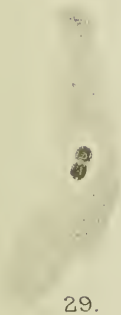
22.

23.



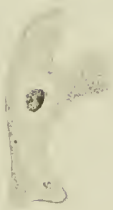
24.

25.



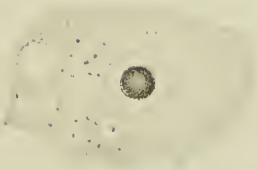
29.

28.



27.

26.



30.

31.

32.

M. Rhodes, del.

Huth, Lith.t London.

AMŒBÆ FROM LIVER-ABSCESS, ETC.

Further Observations on the Cæcal Parasites of Fowls, with Some Reference to the Rectal Fauna of other Vertebrates.

PART I.

By

C. H. Martin, M.A.,
Demonstrator of Zoology at Glasgow University,

And

Muriel Robertson, M.A.,
Junior Assistant to the Professor of Protozoology in the University of London.

With Plates 10–14 and 4 Text-figures.

Table of Contents.

1. General Introduction.

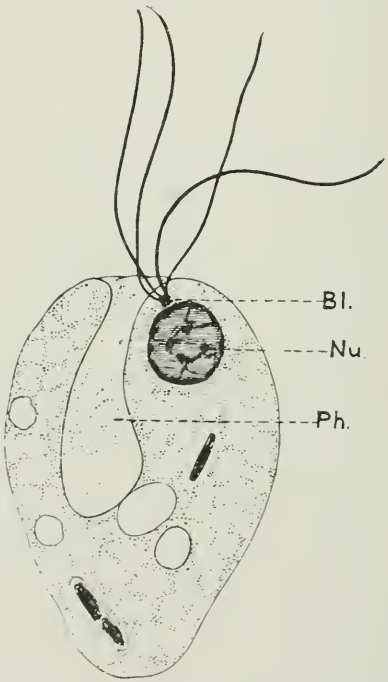
During the last two years we have been engaged in some work on the cæcal parasites of the fowl, and although we do not feel that our work is complete, we have decided to publish some of the results we have obtained.

In recent years there seems to us to have been a tendency to regard the life-cycle of parasitic flagellates as a subject on

which the most hazardous speculations can be put forward, and these, unfortunately, crystallise only too soon into the well-known protozoon life-cycles of the zoological text-book.[1]

Although we do not feel justified in putting forward a complete life-cycle in the case of any of the forms described

Text-fig. 1.



Chilomastix gallinarum, showing the four free flagella, blepharoplast, well-developed pharynx, and nucleus.

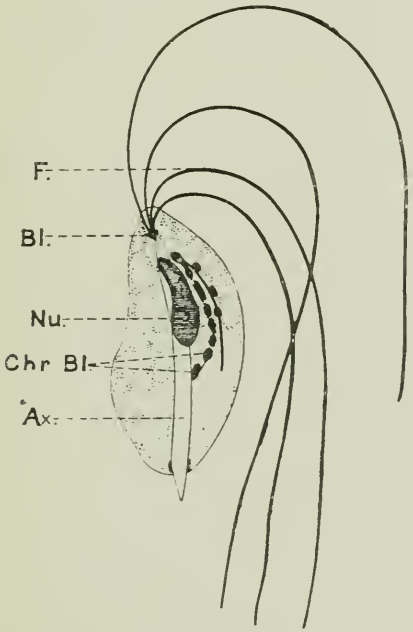
Abbreviations.—*Ax.* Axostyle. *Bl.* Blepharoplast. *b.g.* Basal granules. *Chr. B.* Chromatic blocks. *Chr. L.* Chromatic line. *Cyt.* Cytostome. *F.* Free flagellum. *Ka.* Karyosome. *M.F.* Membrane flagellum. *Nu.* Nucleus. *Ph.* Pharynx.

[1] In this the first part of our paper we only deal with the morphology of the flagellate parasites we have found. In the second part we give an account of the other animal parasites and of the results obtained from new infections.

below, we feel that we are justified in publishing the results obtained for the following reasons :

(1) Our preparations were always made from the wall of the cavity containing the parasite, and not simply from the content of the cavity. Owing, we believe, largely to this fact, we have not only obtained a far more complete series of

TEXT-FIG. 2.



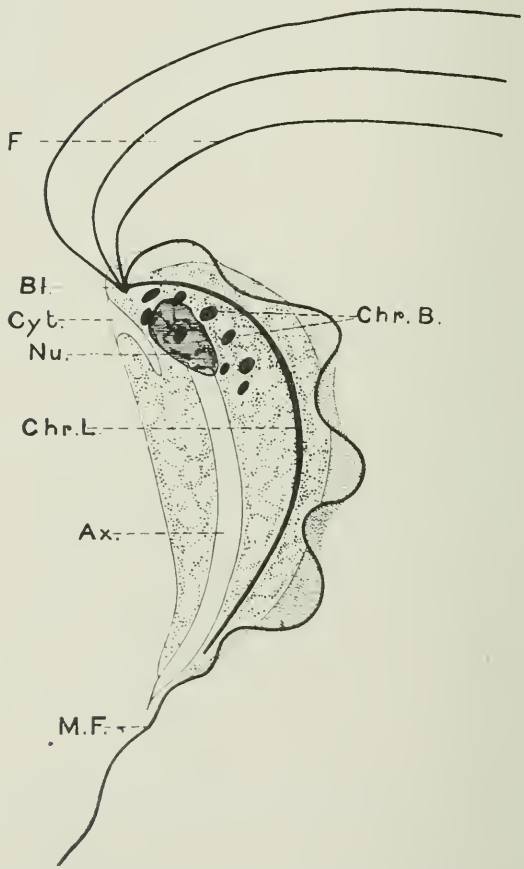
Trichomonas gallinarum, showing four free flagella, membrane flagellum, blepharoplast, cytostome, nucleus, chromatic blocks, and chromatic line.

division of the active forms that seems to have fallen to the lot of the earlier workers on similar forms, but we have also got an interesting series of new stages which must, we are convinced, play an important part in the final construction of the life-cycle of these forms.

(2) Owing to the ease with which it is possible to obtain newly hatched chicks and keep them free from all animal

parasites, we have obtained some really trustworthy data as to the course run by new infections of these forms.

TEXT-FIG. 3.

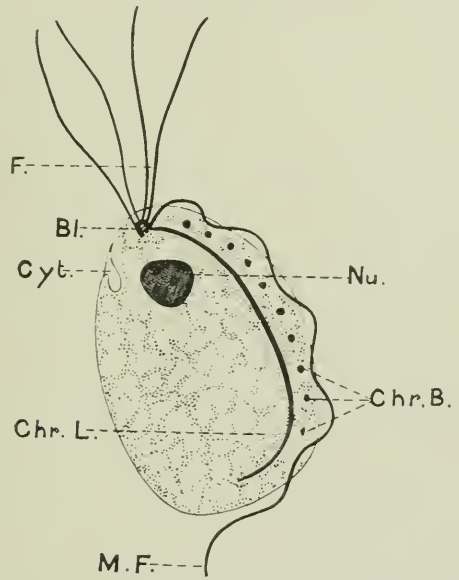


Trichomonas Eberthi, showing three free flagella, membrane flagellum, blepharoplast, cytostome, nucleus, chromatic blocks, chromatic line, and axostyle.

Until, of course, the whole life cycles of these animals are known, the breaking up of the complex series of forms inhabiting the cæca of fowls into good species is a matter of great difficulty. However, it must be remembered that the

same difficulty has to be met in the case of a vast majority of free-living flagellates, and, probably, in that of all the free-living Amœbæ; and partly by means of pure infections obtained in our series of infected chicks, and partly by comparison with similar parasites in other vertebrates, we have obtained evidence which we believe justifies us in dividing

TEXT-FIG. 4.



Trichomastix gallinarum, showing four free flagella, blepharoplast, cytostome, nucleus, chromatic blocks, chromatic line, and axostyle.

the animal parasites found in the cæca of fowls into the following species. [The evidence on which this division is based will, of course, be found in the special part of the paper.]

(1) Chilomastix gallinarum (sp. nov.).
(2) Trichomonas gallinarum (sp. nov.).
(3) Trichomonas Eberthi (sp. nov.).
(4) Trichomastix gallinarum (sp. nov.).

The chief points of resemblance and difference between the

typical active forms of these flagellates are shown in the following table, and the nomenclature that we have adopted for the various parts can be seen in the text-figures.

The literature upon rectal protozoan parasites has in recent years attained such dimensions that it is impossible to give here a detailed historical account of the work done by other observers. Under these circumstances, we have decided to content ourselves with discussing in our final summary the more obvious differences between our results and those of earlier workers on other forms. On the other hand, the literature dealing specifically with the protozoan fauna of the alimentary tract of the fowl is very limited, and as we have already discussed it fairly fully in our preliminary account of these forms, we need here only refer to one or two essential points.

Eberth, in 1862, first described these flagellate parasites from the cæcum of the fowl as "Ein kleines Infusorium," though he did not give them any name.

Stein, in 1878, first put forward the view that these parasites were Trichomonads in which the anterior flagella had been overlooked, and Leuckart, in the second edition of his book, 'Die Parasiten des Menschen,' arrived at the same conclusion. (In his first edition he placed these forms in a new genus—Saenolophus.)

This view of the Trichomonad affinities of these forms was later disregarded by Saville Kent in his monograph of the Infusoria (1881), where the name of Trypanosoma eberthi was given to these flagellates, and in this view he was followed by Bütschli (1889). Most of the later workers on parasitic flagellates — Doflein, Laveran, Mesnil — have regarded these parasites as Trichomonads, but have not furnished any evidence in favour of this view. Lühe, in a short note in Mense's 'Handbuch der Tropenkrankheiten,' has changed the name from Trypanosoma Eberthi to Spirochæta Eberthi, probably on account of Eberth's original figures, in which the only part of the animal that is clearly shown is an undulating membrane. It is evident, we think,

Table of the Main Features characteristic of the Species dealt with.

Name.	Cell body.	Free flagella	Membrane flagellum.	Chromatic blocks.	Chromatic line.	Nucleus.	Axostyle.
Chilomastix gallinarum	Rounded, very well-developed pharynx. Vacuolar cytoplasm	4	—	—	—	Round and vacuolar. The chromatin in part condensed on the nuclear membrane, in part on central masses	—
Trichomonas gallinarum	Rounded. Cytostome small	4	Present	A single line of small blocks parallel to the base of the membrane	Present; runs down the whole length of the body parallel to the base of the membrane	Rounded. Chromatin in small granules throughout the nucleus	—
Trichomonas Eberthi	Carrot-shaped body. Cytostome small	3 long flagella	Present membrane very deep	Large blocks in a double row near the anterior end over the nucleus	Present; runs down the body parallel to the base of the membrane	Elongated oval. Chromatin distributed between a coarse internal reticulus and the membrane	Well developed
Trichomastix Gallinarum	Carrot-shaped body.	4 long flagella	—	Large blocks running half way down one side of the animal's body from the anterior end	Short and inconspicuous; concealed by the blocks	Elongate oval. Chromatin very dense	Present

from the facts mentioned below, that all these names—Saenolophus (Stein), Trypanosoma Eberthi (Kent), and Spirochæta Eberthi (Lühe) must now lapse.

2. Cæca of Fowls.

Before proceeding to the special part of the paper it will be necessary to give a short account of the cæca of the intestine of fowls, since it is this portion of the alimentary tract to which these parasites seem to be confined.

According to Newton ('Dictionary of Birds,' p. 18), "it is highly probable that originally all birds possessed cæca, and that according to the diet these were either further developed or reduced in size, or even lost ultimately."

In the case of the fowl the cæca are two blind sacs, rather variable in size, arising at the proximal end of the rectum. As a general rule the two cæca are found in a distended conditon, and it would appear that almost the whole process of fæces formation in the fowl takes place in these organs. In the early stages of this process the cæca are filled with a light yellowish, rather fluid mass, which appears to be identical with the content of the intestine. In the later stages the content of the cæcum becomes gradually darker and more solid, acquiring at the same time the typical fæcal odour.

It may be well to point out that during early stages of fæces formation the rectum, i.e. the portion of the alimentary tract below the opening of the cæca, is always empty, and it is only in the later stages where the cæcal content has already acquired the characteristic fæcal appearance that anything is found in the rectum itself.

We had hoped that it might be possible to associate certain stages in the parasites with stages in this conversion of the cæcal content into the fæces. In this we have not been successful. On the other hand, there is a very distinct and characteristic change in the appearance of the bacterial flora during this process of fæces formation, to which we will return in a later part of the paper.

There is one interesting point in regard to the cæca of the newly hatched chick at a time before the yolk-sac is completely absorbed and before the animal has begun to take food. In these cases the cæca are periodically filled with a bright green fluid, which gives all the characteristic reactions for bile-pigment. It would seem to us, therefore, that the cæca must play an important part in the economy of the fowl, since even in these young chicks, in which the intestine is practically empty, and the only stuff passing down is presumably the bile, every trace of this is collected for some time in the cæca on its way to the exterior.

3. Methods.

It is a common complaint amongst workers on rectal parasites that division forms are relatively rare: e.g. Dobell, in his paper on the "Intestinal Protozoa of Frogs and Toads," says regarding Trichomastix batrachorum, on p. 209, "stages in division are very difficult to find"; and again, as regards Trichomonas batrachorum, on p. 217, "I have not found so many division stages in Trichomonas as in Trichomastix, but the likeness between them is so great that I have little doubt that they correspond almost identically."

And, in fact, one of the most recent workers on these forms—Bensen, in his paper on "Trichomonas intestinalis und vaginalis des Menschen"—only refers to the possibility of division of the active flagellates in the following passage, p. 122: "Daneben können auf dem Amöboidstadium und im Anfang der Cysten bildung, nach früheren Beobachtern auch im Flagellatzustand, Vermehrungen durch gewohnliche Zweiteilung stattfinden."

This is, we believe, largely due to the fact that the smears used by these workers were made from the rectal content, whereas, as far as can be found in the fowl and the fish which we have examined, the chief dwelling-place of these parasites is in the layer of mucus separating the gut content

from the wall, and, at any rate in the fowl, most of the division stages occur in the actual crypts of the wall itself.

The method we adopted in the examination of our fowls was to open up the cæcum, and after cutting away a portion of the cæcal wall to smear it on the surface of a clean cover-slip. The smears were then plunged into a tube containing either strong Flemming or corrosive acetic.

Both fixatives gave excellent results. The Flemming smears were washed out, according to the method recommended by Overton, with a solution of a little hydrogen peroxide in 70 per cent. alcohol, while the corrosive smears were washed out with a weak iodine solution in 70 per cent. alcohol.

The most serviceable general stains were found to be dilute acid Mayer's hæmalum counter-stained by eosin, though in addition we made use of a large number of preparations with other stains, e. g. iron-hæmatoxylin, Giemsa, Twort, Delafield's hæmatoxylin, borax carmine, alum carmine, safranin, methyl green; and as counter-stains we also found orange G., Licht-grün and picric acid, and picric acid alone, valuable.

Sections were found only to be of value as an indication of the distribution of the parasites in the gut. As has already been stated, the great advantage which the fowl offered over all other animals for the study of these gut parasites seemed to us to lie not only in the great ease with which it was possible to obtain uninfected individuals from eggs hatched in incubators, but also in the fact that it was quite easy to keep the young chicks free from all animal infection for weeks. As far as we are aware none of the other animals which has hitherto been used for work on similar parasites, viz. the frog, lizard, mouse, man, is so easily handled; since, in the first place, with the fowl it is quite easy with suitable incubators to get a practically unlimited supply of uninfected material almost throughout the year; secondly, the animals can be easily kept in a healthy condition on sterilised food; thirdly, the cæca in young chicks are just a convenient size to handle, and they are not too big to prevent the fixed

smears representing a very faithful picture of the actual state of infection. To the results obtained by this method we return in the second part of our paper.

4. Chilomastix Gallinarum.

As will be seen from the table of infected fowls in Part II of this paper, this clearly marked species is a relatively rare parasite of fowls, but in those individuals in which it occurs it is always abundant.

For example, in No. 5, which is one of the chicks obtained from Llanfoist, the cæcal smears are one mass of Chilomastix gallinarum, which was, in fact, the only flagellate present, although neither of the other chicks which were obtained at Llanfoist at the same time showed any trace of the parasite.

In addition to its well-marked morphological characteristics, Chilomastix gallinarum is sharply marked off from the other flagellates in the fowl by two features in its behaviour.

In the first place, the division stages which are so common in the other flagellates, are, even in fowls well infected with this form, relatively rare.

Secondly, the encystation stages, which in other flagellates are extraordinarily rare, are, in the case of Chilomastix, actually common.

Chilomastix gallinarum can readily be recognised in live smears by its relatively large size, its spherical form, and large number of ingested bacteria. It moves on the warm stage fairly rapidly, in a rotary fashion. The body of the active Chilomastix is roughly spherical, though sometimes it is prolonged at the aboral end into a pointed tail. The body measures, roughly, on an average $9\,\mu$ by $7\,\mu$, though larger and smaller forms are to be met with (Pl. 10, figs. 1–4).

From the anterior end of the body there are four fairly large flagella arising from a mass of darkly staining basal granules—the blepharoplast complex. It seems probable that there are four granules in all, and that each flagellum

arises from a separate granule. Near the origin of the flagella is the opening of the well-developed pharynx, which is one of the most characteristic features of this genus, and, as will be shown later, serves as a control in the case of doubtful encysting forms.

The pharynx is roughly horn-shaped, the narrow portion of the horn running back to end in the cytoplasm. The inner side of the pharynx, i.e. the side towards which the nucleus is applied, projects into the lumen of the pharynx as a well-marked rib; the cytoplasm has rather a loosely vacuolar appearance, and in active specimens is crammed with large food vacuoles containing bacteria.

The nucleus in the active animal is very characteristic, both as regards its appearance and position. It consists of a large vacuole containing well-developed chromatic masses, but the greater portion of the chromatin is condensed upon the nucleus wall.

The nucleus lies near the anterior pole of the animal, in close proximity to the opening of the pharynx, so that in the majority of specimens it obscures the origin of the four flagella.

As we have remarked above, we have not been able to obtain nearly as complete a series of divisions in the case of Chilomastix as in that of the other flagellates. Still, as will be seen from the figures of Pl. 10, the main outlines of the process are fairly clear. Division in Chilomastix is clearly transverse to the longitudinal axis of the animal's body.

An early stage is seen in Pl. 10, fig. 5. Here the blepharoplast complex has already divided, and two basal granules, bearing two flagella, have passed to a position near the animal's posterior pole, where there is already an in-sinking, the first sign of the new pharynx. It seems probable that the new pharynx arises as a bud from the old one.

The nucleus has already divided, and it is interesting to note that each of the daughter-nuclei appears to contain the same number of chromatin masses.

In the still later stages of division (Pl. 10, fig. 6) the new

pharynx is well developed, but there is as yet no sign of the outgrowth of new flagella. It is possible that the pointed form (Pl. 10, fig. 2) represents an individual which has just undergone division.

Encystation.—The earliest stage of encystation that we have found is shown on Pl. 10, fig. 7. The form here figured still preserves the rounded shape characteristic of the active individual, but the flagella have disappeared, and the pharynx is still well developed.

In the next stage (Pl. 10, fig. 8) the animal has become much smaller, and at the same time more oval, and a delicate wall has been secreted from its periphery. The remains of the blepharoplast are no longer to be detected, but it has possibly been absorbed into the nucleus, which has now passed from its former anterior to a more central position.

In the next stage the cyst has practically assumed its definite shape. The nucleus has undergone a good deal of shrinkage, leading up to the solid, darkly staining mass seen in the ripe cyst (Pl. 10, fig. 10). The changes undergone in the pharynx during this process are rather difficult to understand. It seems probable that, at any rate in the majority of cases, the thickened lining of the cytostome becomes loosened, and springing out of its original curved shape, lies as a darkly staining line running down one side of the cyst. A darkly staining cap at the anterior pole of the ripe cyst is due to a local differentiation of the cyst-wall. The whole of this process of encystation takes place in the cæcum, and ripe cysts have been found in the fæces some time after deposition. No trace of a sexual process has yet been found in this cycle.

In connection with this form we should like to draw attention to what is apparently a closely allied species, which was found by one of us to be very common in the rectum of the Saithe, Gadus virens, at Millport. It agrees closely with Chilomastix gallinarum, except for the fact that the terminal point is far more developed. We figure this animal in the second part of the paper.

5. Trichomonas Gallinarum.

Trichomonas gallinarum, as will be seen from the table, is one of the commonest flagellate parasites of the fowl.

It is rather variable in size (5·4–7 μ long by 5–6 μ wide). Usually the form is more or less spherical, but sometimes the longitudinal axis of the body is considerably elongated (cf. Pl. 11, figs. 11, 12).

At the anterior end four free flagella arise from a complex blepharoplast, and another flagellum, with a similar origin, passes down one side of the animal, attached to a fairly well-developed membrane, ending freely posteriorly.

Arising from the blepharoplast, and passing down under the membrane is a darkly staining line. This has been termed by Dobell in the case of Trichomonas batrachorum the "chromatic base" of the membrane, but we are inclined to regard this structure as homologous with the line found in Trichomastix gallinarum, and therefore would prefer to use the term "chromatic line." We return to the very important part played in division by this structure in a later part of the paper.

Lying parallel to the line and between it and the base of the membrane there are, in the vast majority of cases, a number of well-marked blocks, which stain readily with chromatic stains. Similar blocks have been found in some species of trypanosomes, Trypanoplasma, Trichomonas, Trichomastix, and Trypanophis.

Near the origin of the flagella there is the aperture of a short curved pharynx. This is not nearly so well marked in this form as in the species which we describe under the name of Trichomonas Eberthi, and can only be seen in favourable specimens.

The nucleus is typically a fairly large oval structure and seems to contain a large quantity of closely packed chromatin granules, but there is a form of nucleus, the significance of

which is not fully understood, in which nearly all the chromatin is condensed on a large central karyosome. This form has been hitherto especially met with in recent infections. A slender axostyle is sometimes found in this form.

Our series of divisions for this form is fairly complete, and we think that it shows clearly that in its early stages division may occur in any one of three planes. These divisions may be termed respectively longitudinal, transverse, and oblique. The determining factors between these three forms of division appear to be: (1) direction of division of the blepharoplast complex; and (2) direction of the outgrowth of the new chromatic line. In all three cases the first process consists in the separation of the basal granules forming the blepharoplast into two moieties, each of which retains two of the original free flagella. The two new blepharoplasts arising in this way are connected by a darkly staining chromatic band.[1] One of the new blepharoplasts retains its old position and its connection with the old chromatic line and the old membrane flagellum. The other new blepharoplast moves away in a direction which is either at right angles or parallel to the longitudinal axis of the animal. From this blepharoplast a new chromatic line quickly grows out, and it will be seen from Pl. 11, figs. 15–30 more clearly than from any lengthy description that the initial plane of division depends largely upon the direction of the outgrowth of the new chromatic line. If, as in the cases Pl. 11, figs. 15–17, the line connecting the new blepharoplasts is at right angles to the longitudinal axis of the animal, and the new line grows out parallel to, and in the same direction as the old line, the division may be fairly described as longitudinal. If the line connecting the new blepharoplasts is parallel to the longitudinal axis of the animal's body, and the new line grows out parallel to, but in the reverse sense to the old line, the initial stages of the division are transverse (Pl. 11, figs. 18–24). If, finally, the

[1] This band has been regarded by other workers on similar forms as the axostyle. We can find no evidence for this view in this form, but we return to this point in our final summary.

line connecting the blepharoplasts is transverse to the longitudinal axis of the animal, as in the first case, but the new line grows out at right angles to the old line, the initial stages of division may be described as oblique (Pl. 11, figs. 25–27).

As regards the details of the division process, there seems to be a complete harmony between all three methods, though at the same time there is clearly a certain amount of time variation in regard to the various details of this process even in specimens undergoing a similar type of division. The general behaviour of the blepharoplast complex and the chromatic line has already been noted above, but it is necessary here to point out that we are completely in the dark as to the manner in which the chromatic blocks get re-arranged in the course of division. There seems to be some reason to believe that in the early stages of division the chromatic blocks become scattered throughout the cytoplasm, but it is quite clear from Pl. 11, figs. 21 and 24, that in the later stages of this process they have again attained their characteristic position under the base of the membrane flagellum.

The new membrane flagellum seems to grow out rather rapidly from the new blepharoplast at some fairly early period in the division process. The behaviour both of the membrane flagellum and the chromatic blocks during division present very sharply marked differences from the behaviour of the similar structures in Trichomonas Eberthi during this process. The two new free flagella in each of the division products grow out as rather thick, black processes from the blepharoplast complex. The stage at which this grows seems to show a certain amount of variability (see Pl. 11, figs. 28 and 26). The band joining the divided blepharoplasts seems to act as a sort of guide to the division of the nucleus, since it is always parallel to an imaginary line joining the two new nuclei; in fact the relation between the band joining the blepharoplasts and the dividing nuclei seems to be a very intimate one, and to have certain points of resemblance to some of the phenomena seen in the mitosis of a typical

metazoan nucleus. On the other hand, we wish to emphasise the fact that there is no evidence for regarding the process of nuclear division in this form as anything but an amitosis. It would seem that in the later stages of division separation of the cytoplasm may proceed more rapidly either at the anterior or the posterior pole, and this would explain such appearances as are shown in Pl. 11, figs. 29 and 30, since here we have what we must regard as two late stages of a longitudinal division, which, however, are clearly not successive. In Pl. 11, fig. 29, the in-cutting is proceeding more rapidly at the posterior pole, with a result that the two individuals will, at a slightly later stage, lie in a head-to-tail position, whereas in Pl. 11, fig. 30, the in-cutting is proceeding more rapidly from the anterior end, and the two new individuals will lie tail to tail.

The last two figures of Trichomonas gallinarum (Pl. 12, figs. 31, 32) are given to show apparent longitudinal division resulting in both individuals possessing the relatively rare type of nucleus with a distinct karyosome. Whether these forms are destined to play some peculiar part in the life-cycle of the animal is a question which must remain for further work. It is interesting to note that in both these cases the forms possess axostyles, which from their position can hardly have played the important *rôle* in nuclear division which has been ascribed to them by some workers.

It will be evident that Trichomonas gallinarum resembles very closely the form described by Prowazek from the lizard.

So far all our attempts at finding any process resembling conjugation have failed. We have found in very large numbers a structure which we must regard as identical with the cyst described by Prowazek, but the cysts by which we find that infection is effected are of a different nature from those described by Prowazek.

To the question of the real nature of this structure we will return in a later part of the paper.

6. Trichomonas Eberthi.

The features which characterise the second Trichomonas form inhabiting the cæca of the fowl have already been mentioned on p. 59, but as in the case of this form we have to deal with a new factor, a rather complicated form change, the equivalent of which has not been met with in Trichomonas gallinarum, we have been forced to divide our description under the following heads:

(1) The typical active Trichomonas Eberthi.

(2) The division of the active form.

(3) The resting stage.

(4) The intermediate stages between the resting and the active form.

The Typical Active Trichomonas Eberthi.

The body of Trichomonas Eberthi is, roughly, of a carrot-shape, measuring, on the average, about 9 μ long by 4–6 μ broad at its widest point.

Arising from the blunt anterior end there are typically three long free flagella and a membrane flagellum, which runs back in connection with a very broad membrane along the side of the animal, to terminate freely beyond the animal's posterior end (Pl. 12, fig. 33). Near the blunt anterior end a small horn-shaped cytostome is frequently found.

The blepharoplast lies near the anterior end, and consists probably of four darkly staining granules, each of which gives rise to a flagellum. Starting from the blepharoplast, and running back along the base of the membrane to the animal's posterior end, there is a very darkly staining chromatic line.

One of the best-marked differences between Trichomonas Eberthi and the previously described Trichomonas gallinarum is given, as was stated on p. 59, by the arrangement of the chromatin blocks. These blocks are here much larger and more definite structures, and are arranged in an

irregular double row near the anterior end. They lie over the nucleus in such a way that if the side of the animal down which the line and membrane run be regarded as dorsal, then the blocks are always on the animal's right side. A large and well-developed axostyle is always present. It arises apparently from the blepharoplast, and after a slightly sinuous course ends in a sharp point beyond the animal's posterior end.

The nucleus is a markedly elongated oval structure, and the chromatin is distributed in a coarse meshwork through the substance of the nucleus, showing usually some tendency to become concentrated on the nuclear wall.

Division of the Active Form.

The process of division seems to be always longitudinal. The first indication of division is usually seen in the division of the blepharoplast complex, but as the two new blepharoplasts may remain in close contiguity during the early stages of this process, the first obvious sign of division is given by the outgrowth of the new chromatic line (Pl. 12, figs. 36, 37).

The new chromatic line in Trichomonas Eberthi always grows out parallel and in the same sense as the old line, and there is no trace of the chromatic band joining the blepharoplasts which was so characteristic of the dividing forms of Trichomonas gallinarum.

The new membrane is formed as an outgrowth down the new line (Pl. 12, fig. 40), but the behaviour of the free flagella during division is a little more difficult to understand.

As has been previously pointed out, the vast majority of active Trichomonas Eberthi bear three free anterior flagella. In the division of these forms it seems probable, from a study of stages resembling Pl. 12, fig. 40, that two of the old free anterior flagella pass in the division of the blepharoplast complex to the moiety of the blepharoplast which gives rise to the new chromatic line, the other free

flagellum, with the old membrane flagellum, persisting in its connection with the other moiety of the blepharoplast. The new free flagella, one in the case of the former individual and two in the case of the latter, are probably formed by splitting from the edge of the membrane flagellum (compare Pl. 12, fig. 34). This splitting process, as will be seen later, is also met with in the passage from the resting to the active stage.

We are inclined to regard the relatively rare stages found with more than three free flagella, and with no other sign of division, as examples in which the flagellar apparatus has undergone precocious development in the transition towards the dividing form (Pl. 12, fig. 35).

One of the most characteristic features in the division of Trichomonas eberthi is furnished by the behaviour of the chromatic blocks. These at an early stage show a tendency to become arranged in a single line parallel to, and apparently in connection with, the axostyle. During the early stages of division there is a marked tendency of the chromatin in the nucleus to become condensed into a number of masses. In early stages the number of these masses seems to be eight (Pl. 12, fig. 40), but in later stages there is a tendency for them to become paired, giving rise to four double masses (fig. 41). Unfortunately we have no figure showing the actual state of the nucleus at the time of division, but it is probable that the latter is a very modified form of mitosis. We are also unable to supply any really conclusive evidence as to the behaviour of the axostyle during division, but an inspection of a large number of stages leads us to believe that the old axostyle undergoes some process of solution, starting from its anterior end, followed by a re-formation in the case of both individuals along the lines of the chromatic blocks (cf. Pl. 12, figs. 41, 42, 43). This hypothesis may seem at first sight rather improbable to those who regard the axostyle as a relatively permanent skeletal structure, but we have so often watched the development of the axostyle in resting forms of Trichomastix gallinarum within a com-

paratively short period that we feel that any objection on this score can carry relatively little weight. On the other hand, we can certainly find no evidence in this form that the axostyle plays the important part in the division of the nucleus which has been assigned to it by some workers on allied forms.

The later stages in division are shown on Pl. 12, figs. 43, 44.

In addition to the active forms of Trichomonas Eberthi, relatively rare true resting forms are also met with.

A typical resting form is figured (Pl. 13, fig. 45), in which the nucleus, blocks and well-developed chromatic line are quite normal, but there is no trace of the flagella. These forms are in life characterised by a peculiar movement, during which a conical wave of protoplasm travels slowly at regular intervals down one side of the animal. Every stage can be found between such a resting form and the active Trichomonas with its three anterior flagella and well-developed membrane. In the form figured on Pl. 13, fig. 46, the membrane is well developed, and in the presumably later stage, figured in fig. 47, an axostyle is also present.

Part of the changes between such a form as is figured in Pl. 13, fig. 48, and the typical active form have been followed on the living animal. The new free flagella are split successively from the edge of the undulating membrane (cf. Pl. 12, fig. 34).

The new free flagella thus formed at first trail behind the animal, and it is to this fact we believe that the difference in movement so often observed in these Trichomonads is due. In the fully developed Trichomonas the free flagella strike successively well out in front of the animal, whereas in the trailing form the free flagella drag behind the animal, and the whole movement of translation is probably due to the action of the undulating membrane.

As to the origin of these resting forms, it would be hazardous in the present state of our knowledge to speculate; but we should like in this place to draw attention to another well-marked but little understood series of forms which

possibly play an important part in the life-cycle of the Trichomonas. These forms possibly represent some conjugation process, but at present we can offer no decisive evidence upon this point. It is, of course, possible that they are degenerate dividing forms. They may be tersely characterised as forms with a double nuclear apparatus and a double chromatic line, but in which the flagellar apparatus is degenerate, and finally becomes entirely lost. In the later stages of this process a number of forms are met with in which the chromatic lines become twisted into irregular circles.

The nuclei of the specimen shown in Pl. 13, fig. 48, would appear to be undergoing division, possibly preparatory to conjugation, and it is interesting to note that forms of this type with nuclei containing only four chromatic masses have been found, e.g. fig. 49. On the other hand, it is possible that the division of such a form as this would give rise to the resting forms we have previously described.

Against this view, however, it may be noted that on one occasion we had an opportunity of keeping a living form of Trichomonas Eberthi, with two membranes and no free flagella, under observation for six hours, during which time there was not only no sign of division, but the animals became more rounded and lost one membrane. It seems very possible that it is to such forms as these that Schaudinn refers on p. 550 in his paper, "Untersuchungen über die Fortpflanzung einiger Rhizopoden" (1903).

"Die in fast jedem Darm vorkommende Trichomonas intestinalis verliert nämlich vor der Kopulation ihre Geisseln und ist dann von einer echten Amöbe nicht leicht zu unterscheiden, weil sie auch mit stumpf-lobosen Pseudopodien umherkriecht."

7. Trichomastix Gallinarum.

The body in the fully developed active form of Trichomastix gallinarum is roughly pear-shaped, measuring about 5 μ long by 3 μ.

From the anterior blunt end four long free flagella take their origin.

Running down one side of the body for a distance of about one third of the animal's length from the anterior end there is a single row of darkly staining chromatic blocks, which seem in most cases to obscure a thin, short, chromatic line. This line is shown in Pl. 13, figs. 52, 53.

The blepharoplast appears to consist of four basal granules, each of which gives rise to one of the four free flagella.

The nucleus is an oval, darkly staining mass lying near the anterior end. Usually the chromatin is scattered more or less irregularly through the substance of the nucleus.

An axostyle is typically present, taking its origin from the blepharoplast, to terminate as a pointed rod at the animal's posterior end. In addition to the elongate active forms, more rounded active forms are also met with in which the axostyle is curved round at its posterior end (Pl. 13, fig. 55). In nearly all these forms, two small darkly staining wedges are found on either side of the axostyle near its posterior end.

We have been able to get a fairly complete series of divisions of the active form of Trichomastix gallinarum, though here again we met with a curious time-variable in the behaviour of the various parts of the divided organism to which we have drawn attention above in the account of the division of the other forms.

The division is here, as far as its initial stages are concerned, a longitudinal one. In the earliest stage of division that we figure (Pl. 13, fig. 57) the blepharoplast complex has divided, two of the basal granules with their two attached flagella have remained in the old position near the anterior pole in connection with the old chromatic line. The other two, with their flagella, have moved to a new position in a slightly posterior oblique direction, and have given rise to a new chromatic line. The two new blepharoplasts are still connected by a chromatic band, which is probably analogous with a similar structure described in the division of Tricho-

monas gallinarum. The axostyle is apparently unchanged, but the chromatic granules are scattered irregularly through the cytoplasm at the anterior end of the animal. The nucleus has become quite round and the chromatin is now distributed between the nuclear membrane and the karyosome-like body lying in the centre of the nucleus.

We have, unfortunately, no stage showing the behaviour of the nucleus at the moment of division, since in the next stage which we figure (Pl. 13, fig. 58) the nucleus has already divided. The two new blepharoplasts with their attached flagella now lie at opposite sides of the animal's body, which has become more rounded. The band joining the blepharoplasts is already showing signs of disappearance, but that this may persist till a much later stage is shown in figs. 59 and 60. The two new nuclei resemble very closely the nucleus of the preceding fig. 57, since in both cases the chromatin is divided in a characteristic way between the nuclear wall and the internal mass. Here, again, in the case of Trichomastix gallinarum, as in the preceding case of Trichomonas gallinarum, we have no definite evidence as to the behaviour of the axostyle during division, but we believe that here also we have a process of solution of the old axostyle and the formation of two new ones.

The chromatin blocks in this specimen are still rather irregularly scattered through the cytoplasm. They seem to show some tendency towards their formation into two more or less parallel longitudinal series. In the succeeding stage, shown in fig. 59, the body of the animal is more rounded, and a new short flagellum is growing out from the blepharoplast in each individual. Each individual possesses a short, fine chromatic line and an axostyle, and the chromatin blocks in each case are showing a tendency to resume the arrangement characteristic of the original active individual.

In the next stage (fig. 60) division has proceeded much further as regards the cytoplasm, but the nucleus is in a condition which we must regard as intermediate between the round form with the chromatin divided between the nuclear

membrane and the karyosome, characteristic of the recently divided nucleus figured and the state shown in fig. 59, in which the chromatin is assuming the irregular distribution through an oval nucleus characteristic of the later stages of division and the normal active form.

In the later stages the line between the blepharoplast has disappeared, and the fourth free flagellum has grown out in the case of each individual. The process of division is in these cases complete except for the severance of the cytoplasm. From the occurrence, however, of free individuals with only three free flagella, it seems probable that in some cases the fourth flagellum is only added after the division of the cytoplasm has been effected.

The aflagellate resting forms of Trichomastix gallinarum are fairly commonly met with, lying usually in groups of three or four, in preparations from the cæcal wall.

In the typical resting forms, such as are figured on Pl. 14, fig. 64, the body of the animal is quite rounded. There is no trace of flagella, but there is a well-marked blepharoplast. The nucleus has the characteristic appearance already described for the active form, but there is a tendency for the chromatin granules either to become massed in a double row or to fuse, forming a darkly staining band round the periphery. An axostyle may be present or absent.

It is probable that the grouping of these resting forms mentioned above is due to antecedent division.

In fig. 65, Pl. 14, a group of three distinct individuals is drawn, of which A is just dividing, whereas B and C are probably the result of a recent division.

If this hypothesis of division be accepted, it is rather interesting to note that in each of the cases figured (Pl. 14, fig. 65), the division of a presumably non-flagellate form has resulted in the production of one individual in which new flagella are growing out, whilst the other individual still remains in the resting stage. A later stage in the outgrowth of the flagella in a form probably derived from the division of a resting form is seen in fig. 66, Pl. 14.

We have been able to observe the transition between the active elongate form and the resting stage in life on a warm stage.

It will be obvious from the account of the dividing resting form given above that it is possible in these cases for the animals to acquire new free flagella as stiff outgrowths from the blepharoplast, a method which, as we have already seen, is adopted by the dividing individuals in acquiring their two new free flagella. In addition to this process we have met with a number of remarkable swathed forms, which we are inclined to interpret as indicating a process of outgrowth of united flagella along the surface of the cytoplasm. Some of the successive stages, which we are inclined to explain on this hypothesis,[1] are shown in Pl. 14, figs. 67–71.

It is only necessary now to refer to such forms as that figured in Pl. 14, fig. 72. Here, again, as in the analogous forms figured (Pl. 13, figs. 49–52) for Trichomonas Eberthi, we possibly have to do with the process of conjugation coupled with a loss of the flagellar apparatus. But here, again, we have no definite evidence to offer in support of this view.

8. EXPLANATION OF PLATES 10–14,

Illustrating the paper by Mr. C. H. Martin and Miss Muriel Robertson upon "The Cæcal Parasites of Fowls," Part I.

[The outlines of all figures were drawn with a camera under Zeiss comp. oc. 12 and 2 mm. apochromat., with long tube at table level. For the study of the detail Zeiss's 1·5 apochromat. with their achromatic condenser Ap. 130 was used.]

PLATE 10.

Figs. 1–10.—Chilomastix gallinarum.

Fig. 1.—Active Chilomastix gallinarum, showing four free flagella, blepharoplast, nucleus, pharynx and ingested bacteria.

[1] These stages might be regarded as stages in the absorption of the flagella: but we have observed the loss of the flagella in the living form on more than one occasion without any indication of these appearances.

Fig. 2.—Tailed form.

Fig. 3.—Rounded form, showing chromatin masses in the nucleus.

Fig. 4.—Very small form.

Fig. 5.—Early division stage of small Chilomastix gallinarum.

Fig. 6.—Later stage of division. Only one of the two flagella is shown in one of the division products.

Fig. 7.—Resting aflagellate form of Chilomastix gallinarum prior to encystment.

Fig. 8.—Beginning of the formation of the cyst wall.

Fig. 9.—Late stage in the encystment of Chilomastix gallinarum.

Fig. 10.—The cyst of Chilomastix gallinarum.

PLATE 11.

Figs. 11-30.—Trichomonas Gallinarum.

Fig. 11.—Active Trichomonas gallinarum.

Fig. 12.—Elongate active Trichomonas gallinarum.

Fig. 13.—Trichomonas gallinarum with axostyle.

Fig. 14.—Active form, showing cytostome.

Fig. 15.—First stage of longitudinal division. The blepharoplast has divided; two basal granules with their flagella remain in connection with the old line and membrane flagellum. A new line has grown out from the other two granules.

Fig. 16.—Later stage in longitudinal division. The new blepharoplasts have moved apart, but are connected by a chromatic band.

Fig. 17.—Later stage of longitudinal division.

Fig. 18.—First stage of transverse division.

Fig. 19.—Later stage transverse division.

Fig. 20.—Still later stage of transverse division. This stage shows the arrangement of the chromatin in the nucleus at the time of division.

Fig. 21.—Later stage transverse division.

Fig. 22.—Later stage transverse division.

Fig. 23.—Transverse division.

Fig. 24.—Probably late stage transverse division; the line between the blepharoplasts has disappeared.

Fig. 25.—First stage oblique division.

Fig. 26.—Later stage oblique division.

Fig. 27.—Still later stage oblique division.

Fig. 28.—Late stage division. A third free flagellum is growing out as a short thick rod in one of the daughter individuals.

Fig. 29.—Late stage division, probably longitudinal.

Fig. 30.—Late stage division.

PLATE 12.

Figs. 31, 32.—Trichomonas gallinarum.

Fig. 31.—Late stage of the division of Trichomonas gallinarum, showing the form with axostyle.

Fig. 32.—Late stage of the division of the form of Trichomonas gallinarum, showing the outgrowth of the fourth flagellum in each of the daughter individuals.

Figs. 33–44.—T. Eberthi.

Fig. 33.—Active Trichomonas Eberthi. Normal form, with three free flagella and membrane flagellum.

Fig. 34.—Trichomonas Eberthi. Form with three free flagella and a fourth flagellum just splitting off from the membrane flagellum.

Fig. 35.—Trichomonas Eberthi. Form with four free flagella.

Figs. 36–38.—Early stages in division, Trichomonas Eberthi.

Fig. 39.—Late stage in division. The chromatin of the nucleus is condensed into eight masses; two new flagella have been formed.

Fig. 40.—Still later stage of division. The chromatin masses have now associated to form four dark masses. The new membrane flagellum is growing out along the new line.

Fig. 41.—Still later stage. The nuclei have divided, though the axostyles are not formed.

Fig. 42.—Late stage division, showing axostyles and divided nuclei.

Figs. 43, 44.—Still later stages in the division of Trichomonas Eberthi.

PLATE 13.

Figs. 45–52.—Trichomonas Eberthi.

Fig. 45.—A flagellate resting stage of Trichomonas Eberthi.

Fig. 46.—Trichomonas Eberthi. Form with membrane, but without free flagella.

Fig. 47.—Trichomonas Eberthi. Form with membrane and axostyle, but no free flagella.

Fig. 48.—Trichomonas Eberthi. Form developing free flagella.

Figs. 49–52.—Doubtful dividing forms Trichomonas Eberthi, perhaps really early stages of conjugation.

Figs. 53–63.—Trichomastix gallinarum.

Figs. 53, 54.—Active elongate form, Trichomastix gallinarum.

Fig. 55.—Active form, Trichomastix gallinarum.

Fig. 56.—Active rounded form, showing chromatic line.

Fig. 57.—First stage in division of the active form.

Fig. 58.—Later stage in division.

Fig. 59.—Later stage of division, showing the outgrowth of a new flagellum in each of the daughter individuals.

Fig. 60.—Still later stage.

Fig. 61.—Later stage, in which the chromatic band between the blepharoplasts has disappeared, and the fourth flagellum has been added.

Fig. 62.—Last stage in division.

Fig. 63.—Trichomastix gallinarum, with only three free flagella.

PLATE 14.

Figs. 64–72.—Trichomastix gallinarum.

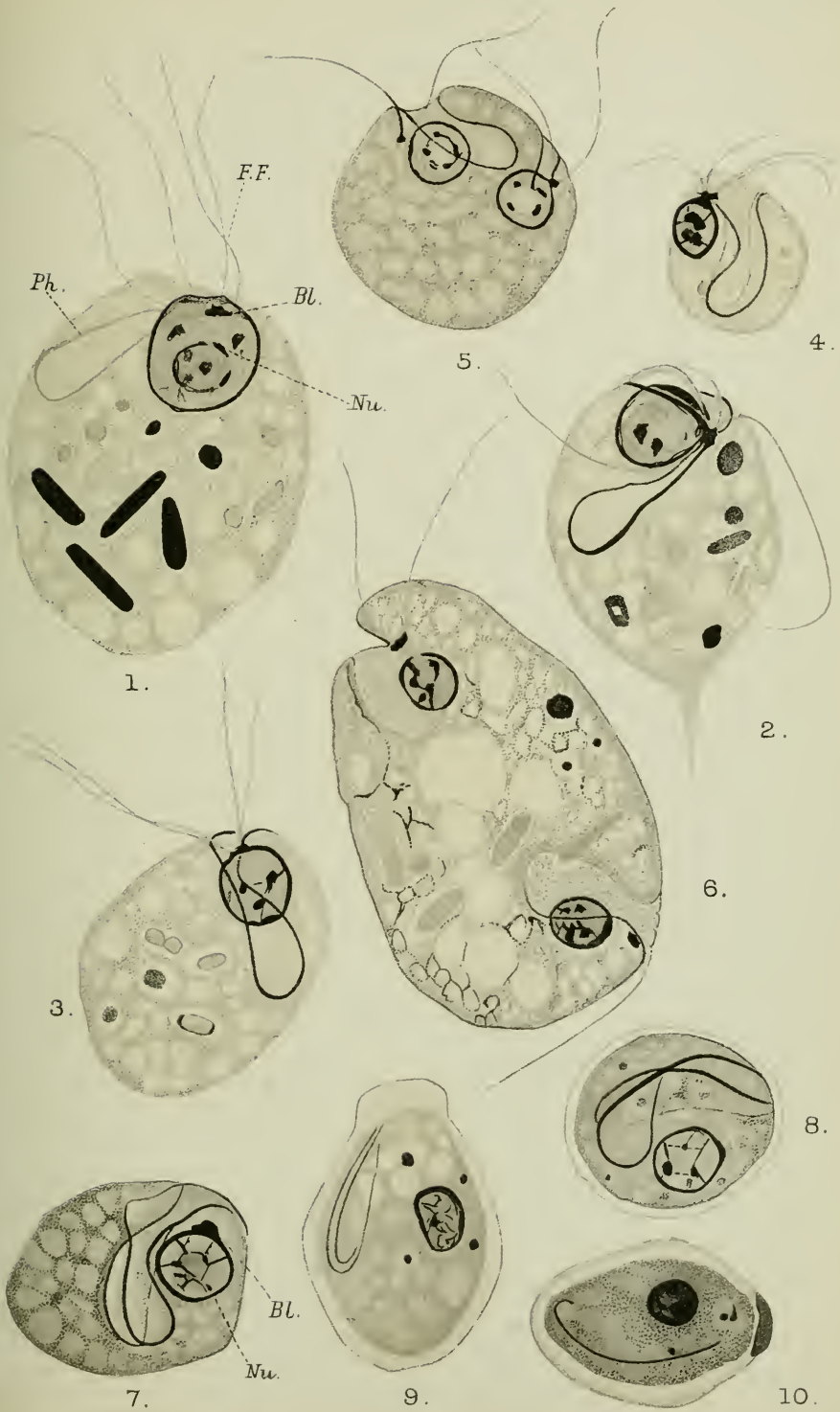
Fig. 64.—Resting aflagellate forms of Trichomastix gallinarum.

Fig. 65.—Dividing resting forms.

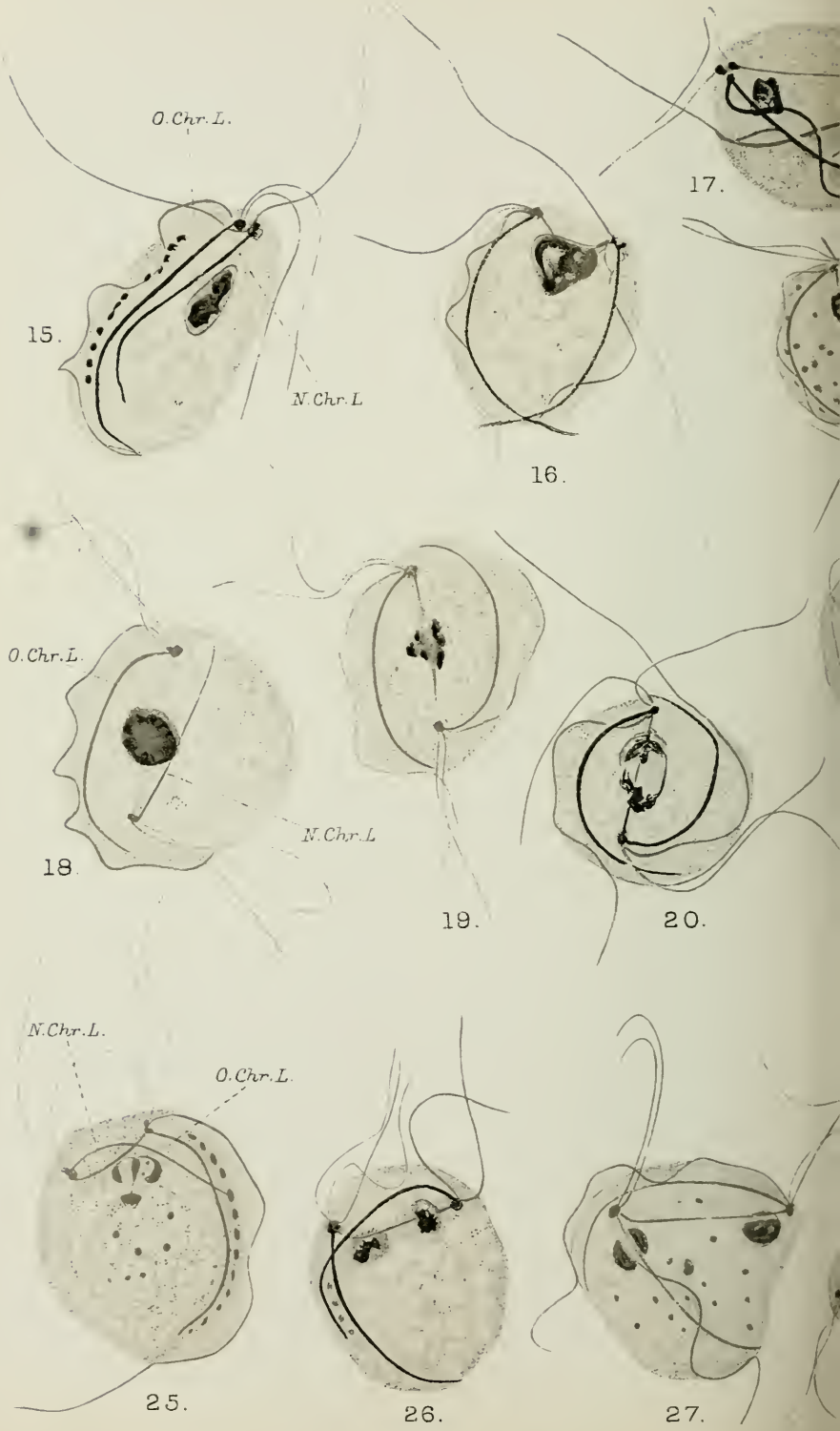
Fig. 66.—Trichomastix gallinarum. Possibly further stage in the outgrowth of flagella in an individual, resulting from the division of a resting form.

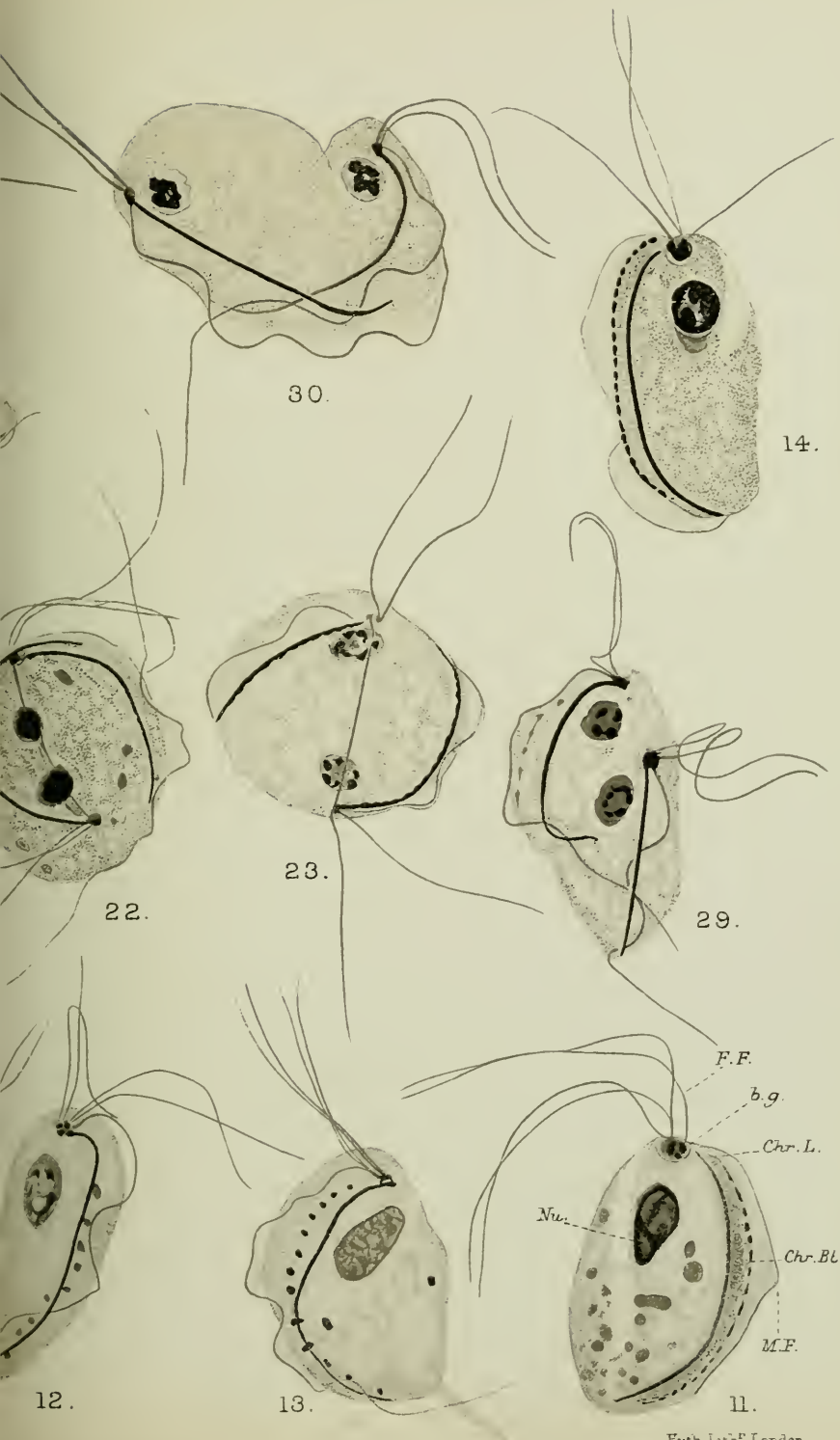
Fig. 67–71.—Possibly stages in the development of flagella on resting individuals of Trichomastix gallinarum.

Fig. 72.—Possibly a stage in conjugation.



Huth, Lith^r London.

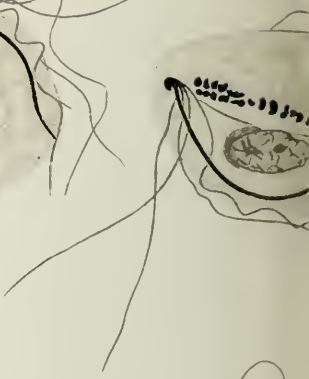

O.Chr.L.
15.
N.Chr.L
16.
17.
O.Chr.L.
18.
N.Chr.L
19.
20.
N.Chr.L.
O.Chr.L.
25.
26.
27.



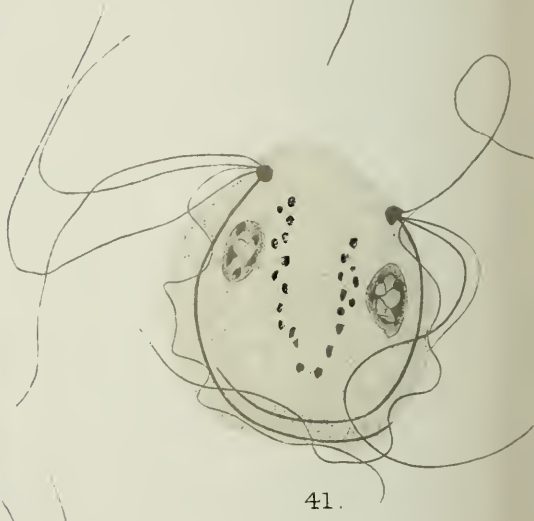
Huth, Lith.t London

31.

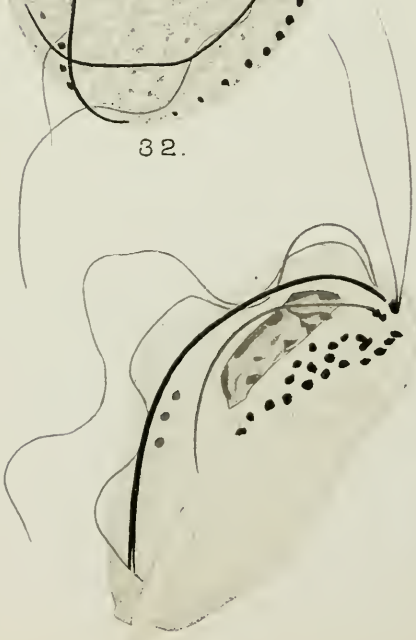
36.



32.

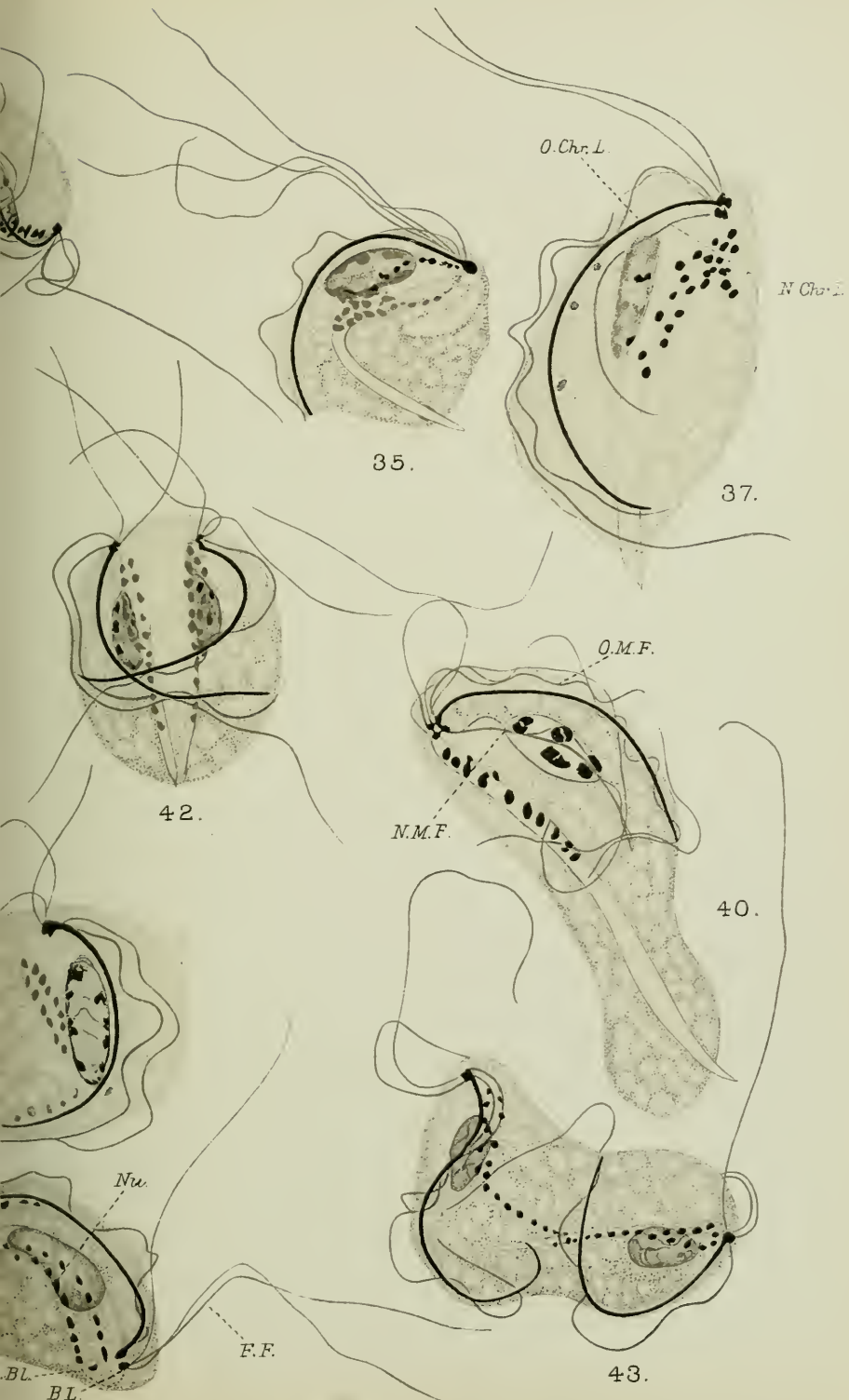


41.



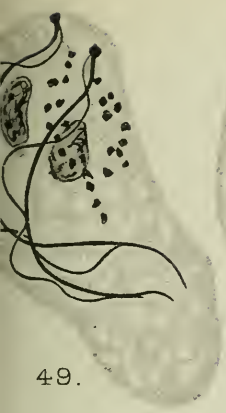
38.

39.



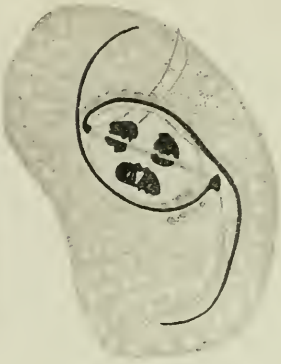
Huth, Lith^r London.


Chr. L.
M.F.
45.
46.
47.
F.F.
Chr. L.
Bl.
Chr. Bl.
Nu.
Ax.
53.
54.
55.
56.
60.
63.



49.

50.



51.

58.

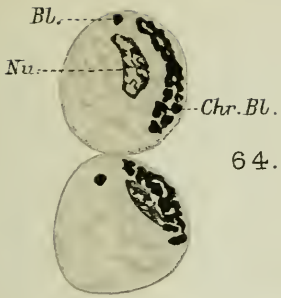
59.

52.

62.

61.

Huth, Lith^r. London.

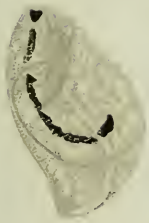


64.

65.

66.

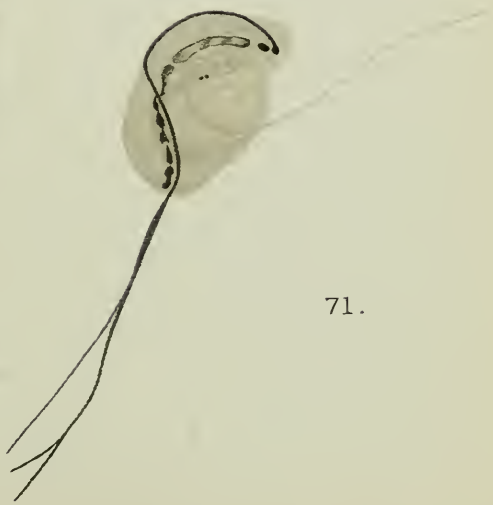
67.



68.

69.

70.



71.

72.

Huth, Lith.ᵗ London.

THE PRESIDENT'S ADDRESS.

SPECULATIONS WITH REGARD TO THE SIMPLEST FORMS OF LIFE AND THEIR ORIGIN ON THE EARTH.

By Prof. E. A. Minchin, M.A., F.R.S.

(*Delivered February 27th*, 1912.)

Gentlemen,—

It is with no slight feeling of diffidence that I venture to discuss the subject which I have chosen for my address to-night, since there is perhaps no problem in the whole range of the natural sciences which appears to be so far beyond the possibility of final and definite solution as that of the origin of life. We must, indeed, "with no middle flight intend to soar" when we attempt to unveil so profound a mystery; we must fit our imagination with wings that can carry it across the profoundest abysses of space or the most immense periods of time. Scientific men are not agreed as to the probable length of time during which living beings have existed on this terrestrial globe of ours; but the lowest estimate is about a hundred million years, the highest about a thousand million. What can the human intellect pretend to know with any approach to certainty about events from which we are separated by so vast an interval of time? All that we can attempt is to discuss the conditions and limitations of the problem with the object of ascertaining what are its possible or probable solutions. I think I need not apologise for bringing the subject of the origin of life before this Club, since the progress, slight though it may be, that has been made towards a solution of the problem would have been impossible without the aid of the microscope.

Before I can approach the main problem, that of the origin of life, it is necessary to discuss first the nature of the living substance, and to gain, if possible, some notion of its simplest or most primitive form. For this purpose I shall be obliged to trouble you with a certain number of hard facts, some of them difficult and abstruse, others perhaps tedious from their

familiarity, which I shall endeavour to deal with as briefly as possible.

In our experience of the natural world there is no sharper distinction to be drawn, with regard to the objects around us, than that between the living and the not-living, between animate and inanimate objects. This distinction is one which is forced upon us from our earliest years; we learn to discriminate between things with and things without life long before we are able to form any exact notion of the distinctive characteristics or properties of living beings. Our conception of life is based on the facts of experience, as that of a property possessed by some things, not by others, and easily lost by those which possess it. Further knowledge, even when based upon exact scientific studies, does not lessen in any way the fundamental distinction between living and lifeless objects. We obtain a clearer and more accurate notion of the characteristic properties of living things, and become able to state with more exactness what objects should be classed as animate or inanimate respectively. But with increase of knowledge the distinction becomes ever sharper, and the gap between the living and the not-living remains as the widest gulf separating any two categories of natural objects, with nothing to bridge it, no transition from the not-living to the living.

Even in the present state of scientific knowledge, it is scarcely possible to frame an exact definition of life in the abstract. It is better not to attempt it, but to inquire merely, what are the principal characteristics of living things, distinguishing them from those that are not living? The popular answer would be, movement, growth, specific form and reproduction according to kind, the offspring being like the parent; to these might be added the peculiar phenomena of sexual behaviour and sexual differentiation, and the possession by many if not all living things of faculties of the kind that we term mental or psychical. Some of these characteristics, however, cannot be applied rigorously for the purpose of distinguishing or identifying living things. Some things that undoubtedly have life do not exhibit movements perceptible to our unassisted senses; on the other hand, there is plenty of movement in lifeless things around us, in air and water. All living things grow, but so do some lifeless things under certain conditions—for example crystals, which

also exhibit specific form. No lifeless things reproduce their kind, but reproduction may be regarded as discontinuous growth, and the offspring produced by a living body is not always similar to the parent, but sometimes very different from it, as in the well-known phenomena of alternation of generations. No inanimate object, however, exhibits sexual characteristics or psychical faculties.

Analysis of the properties of living things shows that their most distinctive characteristic is that which is known as metabolism, signifying that, however distinctive and apparently constant the form and characters of the living body may be, considered as a whole, its substance is undergoing incessant change. A living organism of any kind takes up substances from its environment and causes them to undergo chemical changes which result in their being built up into the substance of the living body itself. At the same time the living substance is also undergoing changes which result in its breaking down, with production, on the one hand, of simpler and more stable compounds than those which constitute the living substance, and, on the other hand, of energy in various forms, such as movement, heat, electrical changes, etc. Hence the principal manifestation of life is the exercise of two processes of change of substance; the one, termed anabolism, is the building up of the complex chemical substances composing the living body from simpler materials; the other, termed catabolism, is the breaking down of these complex bodies in order to generate energy. If the process of building up the body-substance is more active than that of breaking it down, as is usually the case, the result is growth or reproduction. The growth of a living body is therefore quite different from that of a not-living body, such as a crystal, which grows without chemical assimilation. A crystal of salt or sugar, for instance, can only grow in a liquid in which salt or sugar has been dissolved; but to grow a fern in a pot, it is not necessary to supply it with solution of fern, but only with water containing inorganic salts, air and light: then by means of the energy absorbed from the sun's rays the fern is able to absorb the simple substances that it obtains from its environment, to build them up into the complex fabric of its body, and to grow.

Whatever the form or specific characteristics of a living body, this fundamental vital property of metabolism remains its most

distinctive peculiarity, the mark and sign of life. The metabolic process varies infinitely in detail in different cases, but remains throughout the same in principle: a chemical transmutation of substances to build up the complex material of the living body, and the breaking down again of the living substance to produce energy. The process of metabolism may be temporarily in abeyance, but complete inhibition and permanent cessation of metabolism means death; the body ceases to be living and becomes merely a mass of inert and lifeless substance. If a living body appears to us as a stable body, the fact is due solely to the limitations of our senses, which cannot take cognisance of the process of incessant change that is going on. Could we either magnify the substance of the living fabric, or increase the range of perception of our senses to such an extent that we could actually observe the chemical and physical changes taking place, then a living organism, however minute, would appear to us to hum like a factory or roar like a furnace. Chemical molecules are being taken in, broken up, their constituent atoms combined with others to build up huge molecules containing hundreds and thousands of atoms in more or less unstable union. These molecules in their turn are breaking down and smaller groups of atoms are being set free with explosive suddenness, producing heat, movement and other forms of energy.

If now we examine further into the composition of living bodies, we find them to contain universally a substance known as protoplasm, which presents itself as a viscid, slimy fluid, very rarely clear and transparent and then only in parts, but as a rule turbid and containing great numbers of granules and enclosures of various kinds. Some living bodies consist entirely of protoplasm: in others various structures and mechanisms are produced in and by the protoplasm, and by this means a very complex organisation may be produced. The general statement can be made, however, that living bodies consist either of protoplasm alone or of protoplasm combined with the products of its own vital activity. Protoplasm may, in short, be identified as the material basis of life.

When this substance, protoplasm, is examined further, it is found that from the chemical point of view it consists chiefly of substances known as proteins, the most complex chemical substances known. A familiar example of a protein is albumin,

white of egg, but this is a protein of comparatively simple chemical structure; many other proteins are much more complicated in this respect, and contain a vast number of atoms of several kinds combined together, those of most common occurrence being carbon, oxygen, hydrogen, nitrogen, sulphur and phosphorus. Proteins are, in short, chemical compounds of such complex nature that at the present time they are quite beyond exact chemical analysis; it is impossible either to state exactly how they are built up out of their constituent atoms, or to make them artificially in the laboratory. Proteins are characteristic components of living bodies and can only be obtained from living bodies. The enormous chemical complexity of proteins admits of endless variations in them; apparently every species of organism has its own peculiar proteins, different from those of every other species.

There is a further difficulty with regard to the chemical composition of the living substance. The chemist can only deal with it when it is dead; he must begin his analysis by destroying the life. From the dead protoplasm a great number of different proteins are obtained; but it is impossible to state whether the proteins existed separately as such in the living body, or whether they were combined together into a far more complex substance. We cannot say at the present time whether the essential constituent of living matter is a single chemical substance or a mixture or combination of various chemical substances; though from the facts to be considered presently it seems more probable that the second alternative is the true one, and that there is no single chemical substance which could conceivably be isolated in a pure state and be exhibited as the living substance *par excellence*. In other words, the properties of living things are not to be regarded as inherent in one single chemical compound; from a strictly chemical standpoint there is no living substance.

Let us now consider briefly the physical and structural peculiarities of protoplasm. Taken as a whole, protoplasm is distinctly of a fluid nature; it may contain particles or structures of a firm consistence, but this does not prevent it being fluid in the aggregate. Some samples of protoplasm may be less fluid than others; some are very distinctly and obviously fluid, others may be stiffened to a degree that approaches very nearly to the solid condition, lying on the boundary between fluid and solid

matter. The fluid nature of protoplasm is seen from a variety of facts that can be observed without difficulty; chief amongst these are its streaming and flowing movements and the tendency of masses of pure protoplasm to round themselves off and become spherical when they come to rest. Small masses of protoplasm like an amoeba assume a shapeless, irregular and changeable form when in movement; but when their activity ceases from any cause, they become spherical in form. In such organisms a definite, constant and characteristic body-form can be maintained only by the formation of firm structures which act like a primitive type of supporting skeleton, and which have the form either of internal rods or bars or of an external envelope like a skin. A further indication of the fluid nature of protoplasm is seen in the fact that drops of watery fluid, so-called vacuoles, suspended in the protoplasm, tend always to have a spherical form unless there is something to prevent this tendency.

The minute structure and physical nature of protoplasm is a disputed subject which it is not necessary for me to discuss in detail. Any one who has looked at an amoeba under a microscope of even moderate magnifying power knows that its body consists of a fluid ground-substance or *matrix* in which numerous *granules* are imbedded. As regards first of all this matrix, the views of experts are at variance; some consider it to be a homogeneous colloid fluid, but the majority of investigators hold that it consists of two parts, a delicate framework and a watery cell-sap. From the fluid condition of the protoplasm as a whole it follows that both framework and cell-sap must be fluid; they must then be regarded as two fluids which will not mix with one another. The framework is more viscid, and probably consists of albuminous substance; the cell-sap is more fluid, and appears to consist of water containing salts and substances of various kinds in solution.

It is to the granules that I must direct your attention more particularly. In any sample of protoplasm there are a great many different kinds of granules, as shown by their reactions to chemical reagents and stains. Many of the granules undoubtedly represent stages in the process of metabolism described above; that is to say, they are substances which are either on their way to be built up into the complex material of the

living body, or substances which have come into existence by the breaking down of the living matter, and which will be eliminated eventually from it; they may be compared generally either to the fuel or the ashes of a fire. Such granules may be classed generally as metaplastic bodies—that is to say, as bodies which are not, strictly speaking, a part of the living protoplasm itself.

In addition, however, to granules of temporary metaplastic nature, there are certain other granules which appear to be of the greatest importance in the economy of the living substance, since they are of constant and universal occurrence in living organisms of all kinds—namely, the peculiar grains known as *chromatin*, so called on account of their property of taking up certain colouring matters, a peculiarity by which they are generally recognised. In organisms of the most simple type the chromatin-grains are distributed usually throughout the whole protoplasmic body, or the greater part of it; but in the majority of cases they are gathered together at one point, or more than one, to form a structure termed the *nucleus*—that is to say, the kernel, as it were, of a certain mass or lump of the living substance, which is then commonly termed a *cell*. The nucleus varies greatly in structure in different cases, but consists always of a collection of chromatin-grains combined with various accessory substances and structures which may be termed collectively achromatin. The chromatin-grains are the essential element of the nucleus, which never contains any metaplastic bodies of any sort.

When a true nucleus is present, the protoplasm outside it, constituting the cell-body, is commonly termed the *cytoplasm*; it may contain extranuclear grains of chromatin, so-called chromidia, or may be quite free from them. There is no essential difference, however, between the cytoplasm of a nucleated organism and the body-substance in those organisms in which the chromatin-grains are scattered through the protoplasmic body without being concentrated and organised into a nucleus; we may therefore use the word "cytoplasm" to designate the protoplasmic ground-substance or matrix apart from the chromatin-grains, irrespective of whether a definite nucleus exists or not. It is necessary to be clear about the meaning of the terms used, in order to avoid confusion of thought, and in the present

case some writers use the word "protoplasm" as synonymous with cytoplasm—that is to say, to denote the ground-substance of the living matter in contradistinction to the nucleus or chromatin. I think it is best to use the term "protoplasm" for the living substance as a whole, regarding it as composed of two principal parts—namely, the cytoplasmic matrix and the chromatin-grains or nucleus, which, as universal and permanent constituents, are to be distinguished from temporary products of vital activity such as the metaplastic grains.

I must now enter into some further details with regard to the chromatin-grains. The usual test for chromatin is its staining properties, but this is, in reality, a very inadequate method of identification, for two reasons. In the first place, there may be present in the protoplasm formed masses of substances of various kinds which are not chromatin, but which may stain more intensely than the true chromatin, even with the so-called nuclear stains. In the second place, true chromatin often reacts very differently towards the same stain in different cases. A particular method of staining will colour the chromatin of one organism very deeply, that of another not at all. There is no dye known which can be relied upon to stain chromatin always, or which will stain nothing but chromatin in the protoplasm.

From the chemical point of view, all that can be said of chromatin is that it appears to consist of, or to contain proteins more complex than any part of the living substance; the so-called nucleo-proteins, characterised chiefly by being rich in phosphorus-compounds. The infinite variability of the proteins, already mentioned, is seen especially in the case of chromatin. It is highly probable that no two samples of chromatin are ever perfectly similar—a statement which applies not only to organisms of different species, but even to different individuals of the same species.

Chromatin, therefore, is not to be regarded as a substance which can be characterised or defined by chemico-physical reactions or properties. It would perhaps be better to speak of chromatins, or of the chromatin-class of substances, than to use the word "chromatin" in a manner that might convey the idea that a definite chemical compound was meant by it. The conception of chromatin is founded essentially upon its biological properties; it must be recognised and identified by its relation to the vital activities and

life-history, as a whole, of the organism. There are many data both of observation and experiment which indicate that the chromatin-grains are of primary importance in the life and vital processes of organisms of all kinds. In the first place, the chromatin-grains appear to be invariably present in every living organism, and there are some organisms which consist of little or nothing but chromatin. When a cell or a simple living organism reproduces itself by the ordinary method—that is to say, by dividing into two or more daughter-individuals—the chromatin-grains divide also and are distributed amongst the offspring. In many cells the nucleus divides by a very elaborate mechanism, termed karyokinesis or mitosis, which ensures that of the two daughter-nuclei produced, each obtains one of the two daughter-grains of chromatin resulting from the division of each grain of chromatin that was contained originally in the nucleus of the parent-cell. And note this most remarkable fact of all : the sexual process, that great mystery of living matter, consists essentially, in all cases without exception, in plants and animals alike, of union of nuclei or chromatin from two distinct organisms. In the whole series, from man to sea-anemones and Protozoa, what are termed commonly affairs of the heart are in reality affairs of the chromatin-substance. The observed facts of fertilisation and development have led to the belief, I might almost say the conviction, in the minds of many naturalists that the chromatin-grains determine the characters of the offspring and are the bearers of hereditary tendencies and properties. Finally it should be mentioned that the special physiological function of the nucleus in the ordinary life of the cell appears to be that of producing the peculiar substances known as ferments or enzymes, substances which more than any others are characteristic of living bodies and of vital activities.

Equally remarkable are the results obtained by experiment. If unicellular organisms are cut into smaller parts, it is found that any part which does not contain the nucleus or a part of the nucleus may continue living for a short time, but dies sooner or later, and is incapable of feeding or growing and consequently cannot regenerate the lost parts of the organism. A portion of the protoplasm that contains no nucleus may exhibit for a time a certain amount of movement, and may continue to digest food-particles of which the digestion was begun before it was cut off

from the nucleus, but it cannot initiate digestion, nor can it ingest food. Many Protozoa, such as Foraminifera, have shells of complicated structure, and if the shell be damaged the animal repairs the injury; but if its nucleus be removed the power of repairing injuries is lost. On the other hand, if a fragment cut off from a living organism contains the nucleus, or even a portion of the nucleus in some cases, it is able to continue living in a suitable environment, to feed, grow and finally regenerate the entire organism.

From the data obtained from experiment and observation alike, it is clear that the chromatin-substance is of the greatest importance in the life of the organism. Without it the cytoplasm cannot continue to live and cannot initiate the most characteristic activities of the living substance; the processes of assimilation and growth, reproduction and sex, all are dependent alike on the presence of chromatin in the protoplasm and cannot take place without it. Speaking for myself, I believe that it is the chromatin-substance which represents the primary living matter, the true material basis of life, and that the cytoplasm is of secondary importance in this respect.

The objection will doubtless be raised to the view that I have expressed, that, in the case of a unicellular organism, such as an amoeba or a ciliate infusorian, the nucleus isolated from the cytoplasm cannot live by itself and cannot regenerate the body unless a certain amount of cytoplasm be associated with it. This fact becomes perfectly intelligible, however, when we reflect that many organisms in Nature are so adapted and attuned, so to speak, to a particular mode of life that they cannot live except in certain conditions of environment, or unless supplied with special food. It would therefore be in no way remarkable if a nucleus, living always in a particular cytoplasmic environment, were unable to continue living when removed from its natural surroundings. The fact would not, in my opinion, disprove my contention that the nucleus—that is to say, the chromatin—is the primary living substance, any more than the fact that a fish cannot live out of water would be a proof that the water was living as well as the fish.

The most convincing proof, to my mind, that the chromatin is the primary living substance, is that many organisms appear to consist mainly, if not entirely, of chromatin alone, as for example

some bacteria and spirochaetes, and above all the organisms known as Chlamydozoa. I must at this point say a few words about these Chlamydozoa, since they have been discovered or invented very recently—so recently that their very existence is not yet beyond a doubt, and they are not as yet very familiar even to the scientific public.

An acquaintance with the Chlamydozoa has gradually been forced upon scientific and medical investigators on account of the connection which some species of these organisms have with certain very well-known diseases of men and animals—diseases of which the true nature and cause have long been very problematical. Such, in the first place, are small-pox and vaccinia, trachoma and molluscum contagiosum in human beings, and in birds, epithelioma contagiosum and diphtheria. Other diseases possibly attributable to Chlamydozoa are hydrophobia, scarlet fever, measles, foot-and-mouth disease of animals, possibly also distemper, and the silk-worm disease known in Germany as "Gelbsucht." In all these cases the specific virus, different in its properties in each instance, has certain common peculiarities; the pathogenic organism, whatever it may be, is a "filter-passer"—that is to say, it can pass through ordinary bacterial filters without losing its virulence, and it produces characteristic reaction-products or cell-inclusions in the cells of the tissues which it attacks.

As an example of a chlamydozoal organism, I may describe briefly the life-history of the small-pox organism as it is stated by Hartmann, Prowazek and others to take place.* The infection begins with numerous "elementary corpuscles," minute grains barely visible, which can pass through the bacterial filters, and which occur both between and within the cells of the body. Within the cells the elementary corpuscles grow slightly larger, becoming the so-called "initial bodies." Their presence within the infected cell stimulates an abnormal growth of the cell-nucleus, which throws out nucleolar substance into the cytoplasm of the cell. The parasites become enclosed in this nucleolar substance as in an envelope or mantle, hence the name Chlamydozoa. The mantle with the contained parasites forms a characteristic

* See especially Hartmann, *Centralbl. Bakt. Parasitenkunde* (I. Abt., Ref.) xlvii., Beiheft, p. 94; Prowazek, *Handbuch der Pathogenen Protozoen* II. (Leipzig, J. A. Barth, 1911); Prowazek & Aragao, "Variola-Untersuchungen," *Mem. Inst. Oswaldo Cruz* I. pp. 147-158, pls. vii., viii., 2 text-figs.

cell-enclosure known in the case of small-pox and vaccinia as a Guarnieri's body, from its discoverer, who regarded it as the true parasite and named it *Cytoryctes*; according to the most recent investigations, however, the Guarnieri's bodies, and similar bodies in other diseases, are merely extrusions from the nucleus enveloping the true parasites—that is to say, the Chlamydozoa. Finally the Guarnieri's body breaks up, the cell becomes full of initial corpuscles which divide up into numerous elementary corpuscles, and the cycle is complete.

In all this cycle of development the Chlamydozoa multiply actively by simple division into two, and in this process of division there are some noteworthy features. The minute organism is not constricted simply into two, like a bacterium, but becomes dumb-bell-shaped, the two daughter-individuals as they separate being connected by a thread which is drawn out until it snaps. The division recalls that of a centrosome in an ordinary cell. I interpret this to mean that the minute body of a Chlamydozoon is not limited by a membrane like that of a bacterium, but is naked and of a fluid nature; consequently, when division takes place, the viscous body is drawn out in the manner seen and described.

Thus to sum up briefly the characteristics of the Chlamydozoa: they are organisms so minute as to be barely visible, in some cases, perhaps, quite invisible, with the highest powers of the microscope, and to be able to pass through filters which bacteria cannot pass. When seen, they present themselves simply as tiny specks of chromatin, in which no structure can be made out, and their mode of division indicates a structure simpler than that of bacteria, in that at least a membrane enclosing the body appears to be lacking. In all cases known at present they occur as parasites of cells in which they produce characteristic secondary growths or extrusions from the nucleus; although first known as causing diseases of man and the higher animals, they are now stated to occur also as parasites of Protozoa—for example, of *Amoeba* and *Paramecium*—and quite recently the opinion has been expressed that the elusive, and perhaps altogether mythical, cancer-parasite is to be referred to the Chlamydozoa.* To these various characteristics it should be added that

* Awerinzew, S., "Zur Frage über die Krebsgeschwülste," *Centralbl. Bakt. Parasitenkunde* (Abt. I.), lvi. pp. 506–508. 3 text-figs.

Chlamydozoa are difficult, if not impossible, to cultivate on the ordinary bacterial culture-media.

Returning now after this digression to the general problem, it may be said that the more minute a living body, the more it appears to be stripped, as it were, of all cytoplasmic elements and to be reduced finally to chromatin alone. It is, of course, impossible to analyse accurately the structure of the minutest organisms, and statements with regard to them must be made with the utmost caution in the present state of knowledge ; but it can at least be asserted that while many organisms are known which consist mainly, if not entirely, of chromatin, there are none known which consist solely of cytoplasm, and in which the chromatin is entirely absent.

The conclusion which I, personally, draw from the facts which have been summarised briefly with regard to the living substance is that the primary living substance, the *primum vivens*, is chromatin; and from that I draw the further conclusion that the simplest and earliest forms of life were minute particles of chromatin, without other structural accessories, but nevertheless capable of the essential and characteristic activities of living things—that is to say, of assimilation, growth and reproduction by fission. The first steps in the evolution of more complicated forms of life were that these simple chromatin-particles formed other structures around themselves, at first probably in the form of simple protective envelopes, within which the cytoplasmic matrix was gradually developed, until the body was large enough to contain more than one chromatin-grain. This stage of structural complication is practically that seen in bacteria, speaking generally. With further increase of the cytoplasm, proceeding parallel with the concentration of the chromatin into a definite nucleus, the cellular type of organisation is produced, the starting-point for the evolution not only of the vast array of unicellular organisms, but also of all the ordinary animals and plants.

Now I would not have any of you go away with the impression that my views with regard to the chromatin-particles represent orthodox scientific doctrine. On the contrary, I believe that most biologists at the present day would reject my theory most emphatically and would consider me a heretic of the deepest dye for putting forward such suggestions. In conversation, at least, I have never found any one in the slightest degree inclined to fall

in with my views. One objection, which has been put before me by my friend Dr. Chalmers Mitchell, is that in the evolution of the living substance the chemically-simpler may reasonably be supposed to have preceded the chemically-complex, and that if chromatin is more complex than cytoplasm in its chemical constitution, it is probable that cytoplasm was an earlier stage of evolution. Now, admitting, for the sake of argument, that the primary living substance has been evolved from chemically-simpler substances through a series of compounds in an ascending scale of chemical complexity, the question at once arises, at what point in the series was a substance produced which could be termed living? At the bottom of the scale are substances which no one could consider living, such as water, carbon dioxide and inorganic mineral salts; at the top are the complex proteins of the living substance. Are the properties of living matter the result of a continuous physico-chemical evolution from the properties of simple inorganic compounds? Or is life, as we know it, inseparable from a certain degree of chemical complexity, and if so at what point in the series did it come in? These are questions which no one can answer conclusively; all that we can say is that we know of no life that is not associated with chemical substances of the utmost complexity. And we may add further that it is by no means certain that life has been produced by a process of chemical evolution from inorganic to organic; a matter upon which I shall have more to say presently.

The current and orthodox biological view with regard to the primary form of the living substance and of living beings generally is what I may term the cytoplasmic theory, to distinguish it from mine, which I will call the chromatinic theory. According to this view, the cytoplasm is regarded as the primary living substance *par excellence*, of which chromatin is merely a product. The earliest living things were supposed to be formless masses of cytoplasm without a nucleus, Haeckel's Monera. Many naturalists seem to have regarded these hypothetical primitive organisms as by no means minute, not even what we should consider small. Most of us remember, I think, the unfortunate *Bathybius*, which was supposed to consist of primordial protoplasm carpeting square miles of the ocean bed, but which turned out to be a precipitate of calcium sulphate produced by adding alcohol to sea water. Quite apart from a trivial error of this

kind, however, we may safely assert, I think, that the Monera, in the true sense of the word, do not exist. There are certainly organisms in which the chromatin-substance is not organised into a definite nucleus, meaning thereby a body of a certain degree of complexity of structure and co-ordination of parts; but we know of no organisms in which the chromatin-substance is absent altogether. The impression which I derive, rightly or wrongly, from the study of organisms of a simple type of body-structure is that those with abundant cytoplasm, such as amoebae, are far from representing the most primitive type of living beings, speaking generally. Comparison of different forms of life, so far as I am acquainted with them, seems to me to indicate as the most primitive type not a relatively large cytoplasmic organism, but an extremely minute body, a tiny speck of chromatin.

Having now enunciated my views, or perhaps I should say confessed my heresies, with regard to the primary form of life, I will now proceed to discuss its possible origin. I need hardly point out that this is a matter in which it is absolutely impossible to arrive at any certain conclusion. Our data are far too limited in every direction. All that is possible is to indicate the limitations of the problem, to put forward and discuss possible solutions of it, and to consider which of the solutions has, in the present state of our knowledge, the greatest degree of probability. Do not let us forget that a future generation, with increased knowledge and a wider outlook, will probably make merry over our efforts to solve an insoluble problem, just as we are apt to do over the speculations of our forefathers.

At the beginning of this discussion I may lay down two propositions which may serve to confine our speculations and theories within definite limits.

Proposition I.—Life does not originate on our globe at the present time.

This is the well-known problem of spontaneous generation, of biogenesis and abiogenesis. It is a negative proposition, and therefore one which can only be rendered highly probable but can never be proved, speaking from a strictly logical point of view, since a single instance of life originating *de novo* and not from pre-existing life would at once upset the generalisation. The evidence for the truth of the proposition may be summarised briefly as follows: On the one hand, it has often been asserted

that life can originate *de novo*, but in every case the assertion, when critically examined, has proved to be baseless. On the other hand, the experience of many hundreds, even thousands, of investigators, working daily in bacteriological and other laboratories, affords a vast amount of accumulated support for the following statement: When a suitable culture-medium, natural or artificial, which has been previously sterilised, is planted, under proper precautions, with a particular species of organism, that species, and no other, develops in the culture. There is, in fact, such an enormous mass of evidence favouring the belief that an individual organism of any kind whatsoever is the offspring of a parent or parents similar in kind to itself, that the burden of proof rests on those who put forward statements to the contrary. Until, therefore, it has been clearly proved in a single instance that new life can come into being without having arisen from pre-existing life, we are fully justified in assuming that it cannot do so.

Proposition II.—There must have been a period when life did not exist on this earth.

This proposition follows inevitably from a consideration, first, of what is known positively with regard to living bodies; secondly, of the deductions of astronomers and others with regard to the past history of our planet. In the first place, all living things—at least, all those known to us—are extremely sensitive in regard to even moderately high temperatures. Some bacterial spores can survive being boiled in water for a short time, or require a temperature slightly above the boiling-point of water to kill them; but most living beings succumb at a temperature much lower than this, and there is certainly no form of life known to us which would not be destroyed instantly at a low red heat. Yet astronomers assure us that there was a time when our terrestrial globe was incandescent, and it seems as certain as anything can be that no life could have existed on the earth at that epoch.

Taking these two propositions together, that life does not now originate *de novo*, and that there was an epoch in which it could not have existed on the earth, it follows that there must have been some period of past time in which the teeming life of our planet first made its appearance—probably in some simple and primitive form that has given rise by a process of gradual evolution and differentiation to the immense variety of form and

diversity of character which we see in living things at the present time, and which we know also, from the evidence of palaeontology, to have existed through many past ages of the world's history. To put it in one sentence, life on our earth must have had a beginning. If that be admitted, there are then two possibilities—the first that the life known to us originated on the earth itself, the second that it was introduced in some way to our planet from without.

It has been, I think I may say, the view held by the majority of naturalists that terrestrial life originated on the earth itself at an epoch when the earth was cooled down sufficiently to admit the possibility of living things existing on it. This view has been put forward by Sir Ray Lankester. I cannot do better than quote his exact words, as follows:

"A very interesting and difficult subject of speculation . . . is the nature of the first protoplasm which was evolved from not-living matter on the earth's surface. . . . A conceivable state of things is that a vast amount of albuminoids and other such compounds had been brought into existence by those processes which culminated in the development of the first protoplasm, and it seems therefore likely enough that the first protoplasm fed upon these antecedent steps in its own evolution just as animals feed on organic compounds at the present day, more especially as the large creeping plasmodia of some Mycetozoa feed on vegetable refuse. . . . At subsequent stages in the history of this archaic matter chlorophyll was evolved and the power of taking carbon from carbonic acid. The 'green' plants were rendered possible by the evolution of chlorophyll, but through what ancestral forms they took origin . . . it is difficult even to guess" (*Encyclopaedia Britannica*, 9th edition, art. "Protozoa").

If we try to realise in imagination the speculations conveyed in this passage, we may suppose that there was a period when the earth, though far hotter than at present, had cooled down to a certain temperature, sufficiently low for the formation of a firm though thin crust, and to permit of precipitation of water-vapour upon it. The thin crust of the earth was probably continually shrinking, cracking, upheaving, and allowing molten masses to escape from its interior on to the surface; such upheavals, in fact, as we see at the present time on a vastly smaller scale in volcanic eruptions. These cataclysms would cause rapid and

explosive evaporations of enormous quantities of water, which would condense again when and where conditions permitted of its doing so. It is conceivable that the sudden and extremely violent changes of temperature, and the accompanying electrical disturbances, which must have been on a scale exceeding anything with which we are acquainted, would favour chemical decompositions and recombinations to an extent of which we can form no conception in the comparatively peaceful times in which we live; and that in the vast laboratory of Nature there might have been a synthesis and formation of chemical compounds, organic as well as inorganic, such as takes place nowhere in Nature at the present time, such as our chemists have not yet learnt to imitate, or perhaps have not the means of imitating. It is then further conceivable that this period of chaos, of storm and stress on a gigantic scale, might have been the womb of life; that is to say, that by a process of chemical synthesis in Nature, organic compounds might have been formed of ever-increasing complexity, until finally a pitch was reached when a form of matter was evolved possessing those properties and activities which we term vital. Thus might have come into existence the first protoplasm, surrounded by the material for its peculiar property of assimilation, in the shape of various organic compounds of slightly less complexity than itself.

Let us now examine this theory and its consequences a little more closely. In the first place, I need hardly say that we have no means of forming an exact notion of the condition of the earth at that period, nor whether the state of things that I have attempted to reconstruct in imagination would permit of such chemical synthesis as the theory requires. This is a point upon which chemists must pronounce; the naturalist as such cannot attempt to do so.

Turning next to the consequences of the theory, it is to be remarked in the first place that it assumes a chemical evolution of living matter from simple to complex. Consequently the more complex components of a living body, such as the chromatin, would be a later product of evolution than the simpler cytoplasmic elements. The Lankesterian theory of the origin of life would, therefore, favour what I have just termed the cytoplasmic theory of living matter rather than the chromatinic theory. The primitive living matter would be the cytoplasm;

the chromatin would represent one of the many elaborations of the living protoplasm to subserve certain functions. But the theory leads also to a conclusion of the most fundamental philosophical importance. If living matter, with all its remarkable properties and attributes, has arisen by a process of chemical evolution from simple inorganic compounds, then it follows that the properties of living matter differ only in degree, and not in kind, from those of not-living matter. In other words, such an origin of life would undermine and explode the whole basis of the vitalistic conception of life; that is to say, the view that the properties of living things are of a fundamentally different order from those of lifeless things, and that the living is not to be explained or interpreted finally by the physico-chemical properties of the not-living.

Returning now to our primitive protoplasm, surrounded by abundant organic matter for food, let us try to imagine its further history. Our knowledge of living things at the present time would lead us to suppose that the primitive organism would feed and assimilate very actively, growing rapidly in consequence, and then dividing up into smaller masses again; and further, that it would soon tend to become differentiated into various forms under the influence of different environments in different places. The conditions under which it came into being might continue for many ages, generating fresh supplies of food in the shape of organic compounds synthesised in Nature, but in all probability the supply would fall off gradually, since at the present day organic matter does not appear to be synthesised in Nature apart from living things, at least not to any very great extent; such organic matter as is found free in Nature at the present time is probably derived chiefly, if not entirely, from the death and decay of living organisms, or from their excretions. Consequently, our primitive protoplasmic organisms would have to find some new means of getting their livelihood. Some doubtless developed at an early period the animal method of preying upon one another. Fortunately, however, for the continuance of life on the globe, others developed special means and mechanisms for building up their substance by assimilating simple inorganic compounds. There were doubtless many methods of such assimilation, since among bacteria at the present time we find the utmost diversity in the methods of utilising simpler compounds for their growth.

One method, however, judging by its results, seems to have been much more successful, to have paid better, so to speak, than any other—the method, namely, whereby the living substance produced a pigment or class of pigments, sometimes yellow, red, or brown, but most usually green, termed chlorophyll, by means of which it is able to utilise the sun's energy in order to decompose carbon dioxide, and to build up the carbon, together with elements derived from water and inorganic salts in solution, to form the living substance. The organisms which invented, so to speak, this mode of life, flourished exceedingly, and gave rise in process of time and evolution to the entire vegetable kingdom, thereby permitting the evolution of the animal kingdom, depending directly or indirectly for its sustenance upon plants.

If any organisms exist at the present day which represent the original type of living being in its primitive form, unchanged through the ages, it is scarcely possible that they could still exist free in Nature, since they would require an abundant supply of organic nutriment, more abundant, probably, than would be found occurring in Nature. It is probable, therefore, that such organisms would have to be sought among parasitic or saprophytic organisms; that is to say, obtaining their supply of organic matter either from a living body or from one that has lost its life recently.

In contrast with the view that life originated on the earth itself, the suggestion has often been made that the first living things, or the germs of life in some form, were brought by some means to our earth from without. I will not attempt to review or discuss the many theories of this kind that have been propounded; I will confine myself to giving an account of the latest in the field—that, namely, of the famous chemist, Professor Svante Arrhenius,* who has put forward a hypothesis based on grand and wonderful conceptions. His theory starts with the notion that the beginning of life is coeval with that of our universe; that is to say, that "life must always have been in existence, however far back we may carry our thoughts," and that "life itself is eternal, like matter and like energy," so far as our minds can form a conception of eternity. With regard to this assumption, however, it must be pointed out that matter and energy

* *Worlds in the Making* and *The Life of the Universe.* (Harper & Brothers, London and New York.)

are indestructible, and only their form can be changed; while life, as we all know, can be completely destroyed, without passing into any other condition from which it can be resuscitated in its ordinary form.

Arrhenius believes that the life which populates our globe came to it from other inhabited worlds, the means of transport being the so-called radiation-pressure. It has been established by physicists that minute particles of matter below a certain size can be propelled through infinite space by the pressure of rays of light, heat, and all kinds of radiations, and could travel in this way from planet to planet or from star to star. In this way living organisms of a certain degree of minuteness could be disseminated all over the universe and could settle wherever the conditions were favourable. Intensely heated, incandescent bodies giving out powerful radiations, such as the sun for example, would repel them long before they came near enough to be damaged, but on relatively cold planets or heavenly bodies on which the conditions are such that life is able to exist, they could be the starting-point of an evolution of life such as that which has taken place on our globe, an evolution similar as regards its starting-point but not necessarily so as regards its products. This is the so-called doctrine of panspermia, according to which life exists throughout the whole universe in the form of minute germs, capable of further development wherever circumstances permit.

The germs themselves, when floating freely in the interplanetary space, would be subjected to a temperature of about —220° C., a temperature at which all chemical reactions are arrested; they would therefore be in a dormant state, in which all vitality was suspended. They could not therefore undergo any process of multiplication in the interstellar space, and if their numbers were not recruited in some way, the stock of germs floating freely in space would diminish continually and would be absorbed and locked up in those heavenly bodies on which the particles could settle. It is therefore necessary to suppose that the germs can be wafted away from worlds on which they have settled and that other worlds besides ours are inhabited by living things. So far as our solar system is concerned, Arrhenius believes that Venus and Mars probably harbour life, but that Jupiter, Saturn, Uranus, and Neptune

are not sufficiently cooled to permit of the existence on them of living beings.

How would such a germ be enabled to leave our earth and embark on a voyage in space? Arrhenius supposes that a living particle sufficiently minute might get carried by winds and atmospheric disturbances up into the higher layers of our atmosphere until the radiation-pressure of the sun's rays would be sufficient to counterbalance the attraction of gravity, and that then it would be wafted out into space. An organism detached from our earth by the radiation-pressure of the sun would, according to Arrhenius, cross the orbit of Mars after 20 days, of Jupiter after 80 days, of Neptune after 14 months, and the nearest solar system, Alpha Centauri, could be reached in about 9,000 years. In order to undergo the strongest influence of solar radiation, an organism must have a diameter of 0·16 μ—that is to say, $\frac{1}{15625}$ of an inch.

Such, in its main features, is the doctrine of panspermia advocated by Arrhenius. We may now examine it a little closer with regard to both its details and its consequences. I am not competent to criticise it from the point of view of its physical aspects—that is to say, with regard to the theory of transportation of particles by radiation-pressure; that must be left to the physicists and astronomers, by whom, I believe, it is accepted. I can only deal with it as a naturalist. Our first inquiry is naturally concerning the living germs themselves.

As no one has as yet seen a germ of life at its first arrival on our earth, we can only consider what type of organism amongst those known to us would be capable of quitting the confines of our atmosphere and embarking on a voyage in space. It is clear, I think, that no Protozoan cyst, no ordinary seed or spore of any plant, could be carried off our earth by radiation-pressure, because such organisms would be many times too big to satisfy the physical requirements of the theory. Still more is this true of the ordinary visible forms of animal life; the familiar expression "raining cats and dogs" cannot be taken literally, and the Arrhenian theory offers no prospect that any of us will ever be able to pay a visit to Mars or any other distant world. Even most bacteria would be far too large to be carried off the earth, though possibly some of the minuter forms of micrococci, in the state of spores, might undertake the journey. But I am

inclined to think that the only known forms of life which would be capable of such migration are some of the various kinds of "filter-passers," with regard to which our knowledge is slowly increasing, though still very incomplete—namely, those minute forms of life, some of them apparently beyond the range of vision of our highest powers of the microscope, which pass through the bacterial filters. I have dealt with examples of such forms in the case of the so-called Chlamydozoa.

The question arises at this point, what is the size approximately of the smallest bodies that can be seen with our microscopes? I hesitate, before an audience of experts, to pronounce a decided opinion on this point, but I believe that with the best powers of the microscope at present available a body measuring $0{\cdot}1\mu$ ($\frac{1}{250000}$ of an inch) would be just visible, if it were differentiated optically in some way, by staining or refringence, from its surroundings. This is considerably less than the limit of size required by the Arrhenian theory, and an invisible, filter-passing organism would certainly be small enough to be transported by radiation pressure.

So far, then, the Arrhenian theory supports the view that I have put forward above—namely, that the most primitive form of life was a minute speck of substance of the nature of chromatin, since any cytoplasmic organisms known to us, of the type of an amoeba, for example, would be much too large to be propelled by radiation-pressure in space.

In contrast also with the Lankesterian theory, the Arrhenian theory rests on a purely vitalistic basis—namely, on the assumption that living things are fundamentally different in their nature and properties from those that are not living. No generation of the living from the lifeless took place, according to Arrhenius, at any period to which we can throw our thoughts back; if it took place at all, it must have been at a period during or before the beginning of the material universe as we know it. Life carries on its characteristic activities subject to, and restrained by, the physico-chemical laws of matter, but does not owe its origin to those laws, and is not perhaps, in other worlds, bound up with the same forms of matter with which it is connected in ours. The minute chromatin-particle or germ of life might conceivably, on another planet, set in motion vortices of metabolic change quite different from that type with which

we are acquainted here. On the other hand, it follows from the theory of panspermia that all forms of life throughout the universe are related, and it is the opinion of Arrhenius that the activities of life are connected inseparably with the protein-compounds.

Let us now try to imagine what was the fate of the earliest germs of life that, on the theory of panspermia, colonised our globe when it was first in a condition to support life. In the first place it may be noted that the filter-passers of our day show by their activities distinct specific differences amongst themselves. It is therefore possible that more than one variety of living particle came to our planet, and that there may have been some selection amongst those that came.

The first need of the tiny germ, when the warmth of our earth awoke its long-dormant activities, would be food. It is possible that some of these heavenly visitors may have acquired already, in another world, the power of constructing organic matter from inorganic; but it is more probable that a supply of organic matter was a necessity for them, since all the filter-passers and Chlamydozoa known to us at present are parasites. We may suppose, therefore, with Sir Ray Lankester, that a certain epoch of the earth's history was favourable to the synthesis of organic matter of at least a certain degree of complexity; and that thus a supply of food was provided upon which the Arrhenian germs were able to make a start. In any case they must have multiplied rapidly, adapted themselves to various modes of life, and given rise by natural evolution to the various forms of life on our globe through an immense period of time. Arrhenius considers that the interval of time between the first appearance of life on the globe and the Cambrian epoch was at least as great as that from the Cambrian epoch to the present day.

In the preceding paragraphs I have tried to set forth critically the two opposed theories of the origin of life, one or the other of which must be true so far as its main thesis is concerned; that is to say, life must either have originated on the earth or have come to it from without. He who would attempt, however, to judge and decide between these two possibilities would be indeed a bold and a rash man. Scientific knowledge of living things is at present much too incomplete in at least two directions to render any such judgment even approximately just. We require

a much more exact knowledge of the physical and chemical nature of the living substance and its activities; and we are as yet very ignorant with regard to the simplest forms of life, their occurrence, species, activities and structure. I will only attempt, therefore, to consider some of the consequences of each theory.

On the Lankesterian theory, we can understand why life does not now originate on the earth under ordinary circumstances, natural or artificial; and it follows from the theory that all life known to us has a common origin from a form or forms called into existence at a particular epoch under special circumstances. But if life has been generated from lifeless matter at any time, it should be possible, in the abstract at least, to imitate and repeat the circumstances under which it arose, were they known to us, and thus generate life again.

On the Arrhenian theory, life may have started its development and evolution many times on the earth, and fresh germs may be falling on the earth now. The statements of some of those who have positively affirmed the occurrence of spontaneous generation might be explained, conceivably, by supposing an Arrhenian germ to have fallen into their cultures, though for my part I am much more inclined to attribute their results to untidy and inexact technique.

In short, if life was generated on the earth, it should be possible to generate it again; and if new germs of life are coming to the earth continually, it should be possible at some time to intercept them and examine them; but whether either of these possibilities is capable of ever being realised is more than any man can say.

The two theories bring us down, as I have pointed out, to the bed-rock of philosophical speculation, to the two opposed standpoints of vitalism and mechanism, the difference between which may be illustrated by a fictitious example. Suppose it were possible to imitate artificially the structure and fabric of a living organism, say of some plant, to such a degree of exactitude that the mimic resembled the model not only in the minutest details of structural arrangement of all its parts, but also in the chemical composition, molecule for molecule, in each corresponding part. Would then the mimic have the same properties as the model—that is to say, would our artificial plant be living? Without hesitation the mechanist answers Yes, the vitalist No. To the

mechanist, life is the sum of the chemico-physical properties of the various forms of matter composing the living body; to the vitalist, life is something more than that, something which utilises chemico-physical properties as a workman uses his tools, but which is distinct from them.

Since it is impossible to put the matter to a crucial test, each of the two opposed views remains a pious belief merely. For my part I believe that the view which a man holds with regard to the nature of life depends on the inner constitution and fabric, so to speak, of his mind, and not on the reasoning process. A man is born a vitalist or a mechanist before ever he has thought about such matters, and to argue on the subject is futile. At a time when I was younger than I am now, I have myself debated and discussed such matters hotly; like old Khayyám—

> Myself when young did eagerly frequent
> Doctor and Saint, and heard great Argument
> About it and about: but evermore
> Came out by the same Door as in I went.

It is my present belief that all that is gained by such discussions is to enable a man to ascertain what is the type of mental bias with which he has come into being. The questions which lie at the base of the difference of opinion are at present not capable of being put to the test; and so far as one can see, they seem likely to remain for ever the most inscrutable of problems.

Reprinted from the British Medical Journal, *August 19th, 1911.*

ON THE OCCURRENCE OF AN INTRACELLULAR STAGE IN THE DEVELOPMENT OF TRYPANOSOMA LEWISI IN THE RAT-FLEA.

BY

E. A. MINCHIN, M.A., F.R.S.,

PROFESSOR OF PROTOZOOLOGY IN THE UNIVERSITY OF LONDON

AND

J. D. THOMSON, M.A., M.B., C.M.

(From the Protozoological Laboratory, Lister Institute.)

(Preliminary Note.)

In studying the early phases of the developmental cycle through which the rat-trypanosome passes in the rat-flea, we have found that the trypanosome penetrates into the cells of the epithelium lining the stomach (mid-gut) of the flea, and there goes through a process of multiplication. Since no such phase has been observed hitherto, so far as we are aware, in the development of any trypanosome in its invertebrate host, and since, moreover, this discovery may throw some light on certain peculiar features of the development of the pathogenic trypanosomes, we consider ourselves justified in bringing forward a brief account of our observations, so far as they have gone, without further delay, hoping to give later a full and detailed account, with figures, of this and other phases of the development.

The intracellular phase of the trypanosome has been seen by us in fleas taken from the breeding-cage, not previously exposed to infection, and fed on infected rats during a known period of time, as early as twelve and as late as thirty-six hours after feeding; but our observations are not yet sufficiently extensive to enable us to place precise limits of time to the duration of this phase. We can only say at present that we find it most abundant about twenty-four hours after the flea has taken up the trypanosomes from the rat, and that it occurs in all parts of the epithelium of the stomach, from close behind the proventriculus to the pyloric region; that is to say, over the whole extent of the true mesenteron or mid-gut, lined by the embryonic endoderm or hypoblast. In this region

of the digestive tract the epithelium, as is well known, does not secrete a chitinous cuticular lining to the gut, as in the regions before and behind (stomodaeum and proctodaeum).

The actual penetration of the epithelial cell by the trypanosome has not been observed by us up to the present, but trypanosomes of quite ordinary appearance, which have, apparently, effected an entrance very recently, have been seen frequently within the cells. The earliest change which such a trypanosome undergoes is that the body assumes a bulbous form which may be compared to that of a pear with a stalk, or of a tadpole with a long tail. The pear-shaped body is easily seen to be produced by the doubling on itself of the posterior two-thirds or three-fourths, approximately, of the body of the trypanosome, while the anterior portion of the body, with the flagellum and undulating membrane, forms the stalk or tail. In stained preparations it is seen that the folding-up of the body brings the kinetonucleus in front of the trophonucleus; the result is a flagellate of pseudo-crithidial form, easily distinguished from the true crithidial forms both by the fold of the cuticle running down the middle of the body, and by the fact that the flagellum does not pass straight to the kinetonucleus along the undulating membrane, but curves round the rounded posterior end of the body to run forward (apparently) to the kinetonucleus. Seen in the living state, these pear-shaped forms appear slightly flattened, as is to be expected from their mode of formation; they are in incessant movement within the cell, exposing now their flat surface, now their edge, to the view of the observer; and the flagellum and undulating membrane can be made out plainly, as a rule, giving an appearance as if the stalk of the pear tapered to a fine point, but in a few cases the stalk appears of even thickness, and evidently consists of the flagellum alone in the early stages. The body itself is far from being fixed and rigid in its contours; on the contrary, it shows continual changes of form of the kind that are termed, in the Flagellata generally, "metabolic," as distinguished from true amœboid movements, form-changes due to the incessant, restless movements of a contractile, protoplasmic body, within a thin, yielding envelope or cuticle. In preparing the stomach wall for microscopic examination it frequently happens that epithelial cells are ruptured or burst by the pressure of the cover-glass, setting free the trypanosomes, of which there may be several, four or five, within one cell. Then these pear-shaped forms, if watched carefully, can be seen to uncurl themselves and assume the form of free trypanosomes; sometimes they curl up again and uncurl several times, swimming actively the while.

Within the cell the pear-shaped forms described in the last paragraph are seen to grow in size, becoming at the same time more uneven and irregular in their contours, so that the form of the body can only be described as block-like; the outline, so far as it is possible to describe contours which are changing incessantly, is rounded or

squarish ; the " tail " is distinct, tapering from a relatively broad base to a fine point, and always in motion; the metabolic form-changes proceed without interruption, so that the contours of the body undulate and change every moment, and the body as a whole assumes various shapes, often returning to the pear-like form for a brief space of time. The block-like forms grow until they reach a size relatively very large. In some cases they appear to be lodged in a more fluid, but not sharply delimited, portion of the cytoplasm of the host-cell ; in other cases they appear to be within a vacuole limited by a distinct wall or membrane, and in many cases the vacuole is set free by rupture of the cell in making the preparation. Free vacuoles of this kind exhibit a degree of stability and persistence which indicate that the host-cell may secrete an envelope or capsule of some kind round the intruding parasites, of which there may be one, or several in various stages of development, in each such vacuole. When the block-like forms become of large size, the movements of the flagellum cause them to revolve and rotate within the cell in an irregular, jerky manner. It can be seen clearly that forms moving in this manner push and bump against the cell nucleus and against other forms present in the cell and cause them to move also, showing that they come into actual contact with them.

When the block-like forms have reached the limit of their growth, changes take place in them which cannot be followed in full detail in the living condition, but which can be seen to consist in a division of the large block-like body into a number of daughter trypanosomes by a process of multiple fission which goes on within the periplast of the parent form. By the time that the block-like form has attained its full size its nuclei are seen to have divided into a number of daughter-nuclei ; and while still agitating its flagellum and changing its contour, the body is seen to exhibit a striated appearance in its interior. Very soon after this a marked change takes place ; the body becomes spherical, and ceases to show metabolic form-changes; the tail disappears altogether, and the sphere as a whole becomes almost motionless, except for slight oscillating or trembling movements due to the increased agitation of its contents. When carefully examined, it is now seen that the spherical body contains in its interior a number of distinct trypanosomes, squirming and wriggling over each other in the confined space like a bunch of eels in a sack ; to their movements are due the oscillatory movements of the sphere, which since the disappearance of the tail can perform no movements of itself. The enveloping membrane of the sphere is seen to become more and more tense, the movements of the contained trypanosomes ever more active and vigorous, and everything betokens the approach of the crisis, which comes finally with startling suddenness; the sphere bursts in an explosive manner and liberates a mass of perfectly-formed trypanosomes. The explosion can be seen best in spheres which have been set free accidentally

from the host-cell in the process of manipulation; then the sphere is seen to burst suddenly, and the trypanosomes scatter in all directions. Under normal conditions, however, the spheres burst within the cytoplasm of the host-cell, and the liberated trypanosomes are seen moving actively about for some time within the cell, from which they escape one by one. In one case the formation of three spheres within the same cell was observed; each sphere grew to its full size, burst, and liberated its bunch of trypanosomes within the host-cell, first one sphere and then another.

It is difficult to estimate exactly the duration of the block-like and spherical stages, because when the parasites are under observation between slide and cover-glass their vitality is soon impaired, and they gradually become moribund. From some of our observations we received the impression that the intense illumination of the field of the microscope is injurious to them. Some parasites under observation were seen to become moribund, and at last perfectly quiescent, but on moving the slide others were found in other fields quite active; and, when these died off in their turn, active forms were found in a third field on the same slide. From a number of cases that we have observed, and especially from those which have been watched when quite freshly put up, it is clear that the final spherical stage may last in some cases from about a quarter to half an hour, but in other cases for a much shorter time, not more than a few minutes. The following series of observations, jotted down by one of us at the dictation of the other, who was watching through the microscope, may serve as an example of the changes observed and the times they take to fulfil themselves:

12.13. An active sphere was seen within a cell, evidently full-sized, but still metabolic; it revolved with jerky, irregular movements by means of the flagellum, which was clearly seen; its contents appeared to be divided up or in process of division.

12.26. Condition much as before, but the contents appeared to be more distinctly divided up, and the daughter-individuals to be performing independent and separate movements, squirming over one another like eels; movements of rotation and metabolic form-changes still continuing; flagellum still visible.

12.32 Flagellum still visible.

12.36. Revolving movements and metabolic form-changes much slower; flagellum not seen.

12.37. Form more perfectly spherical, alterations in contour less marked, due apparently entirely to the very active movements of the contained trypanosomes, which could be seen very clearly; no rotating movements; flagellum looked for carefully, could not be seen.

12.40. Contour of sphere tense, almost rigid.

12.41½. Burst!

The trypanosomes set free by the bursting of the spheres are normal forms, complete in all respects, and of a size equal to, or slightly exceeding, that of the ordinary *Trypanosoma lewisi* of the rat's blood; the undulating membrane extends down the body to the neighbourhood of the kinetonucleus, which is situated near the posterior

end; briefly expressed, these forms are perfectly trypaniform, and not the least crithidial, as regards their internal structure. Their shape and movements differ slightly, however, from those of the ordinary blood-trypanosomes; the body is stiffer, less sinuous in its movements, slightly swollen towards the posterior end, which is sharply pointed; the anterior end of the body tapers very gradually, and the flagellum is long. When moving freely in the fluid medium they travel fast with the flagellum directed forwards; but they may be seen frequently with the hinder end of the body in contact with the epithelium, the flagellum undulating rapidly, and giving the impression that they are butting against the cells with their hinder ends, which may possibly be the manner in which they penetrate and enter the cells. As regards the number of trypanosomes liberated from a sphere, it is difficult to be certain; in the living state we have frequently tried to count them, and came to the conclusion that the number was about eight; but in our stained preparations we have found in one case a sphere containing thirteen couples of nuclei, each couple consisting of a trophonucleus and a kinetonucleus; and in another case there were eighteen trypanosomes in a bunch, which appeared to represent a sphere recently burst. The number produced would appear, therefore, to be irregular and indefinite, and this is borne out by observations on the living objects, since we have seen a very small sphere burst in one case, and in another we saw a sphere of very large size, far beyond the usual dimensions. We have the impression, however, that the normal or average size of the sphere is one that would liberate eight trypanosomes, and that this may be taken as the normal standard of capacity, so to speak, of the sphere, but that it may sometimes be much less or much greater.

The above account of the intracellular multiplicative phase is founded almost entirely on observations made by us on living material; it amounts, summarized briefly, to the following: Trypanosomes penetrate into cells of the epithelium of the stomach, where, retaining their flagellum and undulating membrane, they double upon themselves and grow to a large size, performing metabolic movements and continually altering their form the while. They gradually round themselves off until a spherical stage is reached, when the flagellum and undulating membrane disappear and the metabolic form-changes cease; meanwhile the contents of the body within the periplast have divided completely into a number of daughter-trypanosomes which writhe about and twist over each other within the maternal periplast-envelope like a bunch of eels. The envelope becomes more and more tense, and finally bursts with explosive suddenness, setting free the daughter-trypanosomes, which are perfectly formed and of full size, and escape from the host-cell by their own activity.

This development is less easy to follow in full detail in stained preparations, chiefly on account of the difficulties of technique. It is not easy to get good preparations of the intracellular phases while still within the cells, and

even when they are set free the spheres are difficult to stain properly; either they appear in the preparations as opaque masses, if the stain is insufficiently extracted, or on the other hand too much stain may come out, and details of structure are lost, especially as regards the flagellum, which may become invisible in consequence. We have, however, confirmed in our preparations all essential points of the development described above, and we hope soon to obtain a complete series of the stages of the development.

We defer also to a later communication a number of questions which naturally arise with regard to this developmental phase, the first of which is: How is this intracellular multiplicative stage related to the developmental cycle, as a whole, of the trypanosome in the flea? We are not prepared to discuss this point fully at the present moment, but from all that we have seen we are inclined to think that this phase marks the beginning of the development—that it is, in short, the first act, so to speak, of the cycle within the flea. The intracellular phases are by no means to be found in every flea examined twenty-four hours after it has fed on an infected rat. In the majority of the fleas the trypanosomes have disappeared altogether at this period, though in those in which trypanosomes are present there are generally intracellular stages to be found. This result corresponds with our experimental results, which show that only a small proportion of fleas fed continually on infected rats acquire a permanent infection with the trypanosomes whereby they become themselves infective to rats in their turn. We are inclined to think that whether the trypanosomes succeed in establishing themselves in the flea or not depends upon whether they succeed in penetrating the cells of the stomach wall and going through the intracellular multiplicative phase here described. In the majority of the fleas the trypanosomes taken in appear to be digested together with the blood, and never to succeed in establishing themselves in the flea or in going through their developmental cycle; only in a relatively small number of cases do the trypanosomes resist the digestive juices of the flea and succeed in holding their own. Analogous conditions have been observed by Kleine, Bruce, and others in the development of pathogenic trypanosomes in tsetse flies; only in a relatively small proportion of the flies fed on infected animals do the trypanosomes go through their full developmental cycle.

It seems to us further possible that the facts observed may also throw some light on another peculiarity in the development of pathogenic trypanosomes that has been noted by all those who have studied them. It has been observed in the development of *T. gambiense* in *Glossina palpalis*, for instance, that the trypanosomes disappear from the digestive tract of the fly at a period some five to seven days after they have been taken up; in a few flies they reappear again later in the digestive tract in enormous numbers. This temporary disappearance of the trypanosomes from the gut of the fly may perhaps find its

explanation in the trypanosomes undergoing some multiplicative stage, similar to that seen in the case of *T. lewisi*, in the cells lining the digestive tract. We have written to our colleague, Miss Muriel Robertson, who has recently gone to Uganda for the purpose of studying the development of the pathogenic trypanosomes, informing her of the developmental phases that we have observed in the flea, and suggesting that similar phases may perhaps occur in the development of trypanosomes in tsetse flies.

A further question which arises, with regard to the intracellular phase described above, is whether it occurs as a single generation in the developmental cycle of the trypanosome, or whether there may be several successive generations of intracellular multiplication, before the development takes its further course. This is a question to which it is difficult to give a definite answer, since it is scarcely possible to keep the trypanosomes alive between slide and cover-slip long enough to observe more than one generation of the parasites. Not only the trypanosomes, but the epithelial cells also, soon degenerate and become moribund under such conditions. We are inclined to believe, however, that trypanosomes which have been liberated by the bursting of a sphere within a cell may, after becoming free from the host-cell, penetrate into other epithelial cells and repeat the process of multiple fission by which they themselves came into being; in other words, that several generations of intracellular multiplication in the stomach succeed each other in the beginning of the development of the trypanosome in the flea.

Intracellular stages of trypanosomes have been described by many investigators in that part of the life-cycle which takes place in the vertebrate host. Without multiplying instances or referring to the many cases, some of them very debatable, in which the occurrence of such stages has been alleged, it will be sufficient here to refer to the multiplication of *T.* (*Schizotrypanum*) *cruzi*, the parasite of human trypanosomiasis in Brazil. According to Chagas,[1] the parasite goes through a process of multiple fission in the capillaries of the lung in the main as follows: A full-grown trypanosome loses its flagellum and becomes folded on itself, first into the form of a crescent, which by fusion of its two horns becomes an oval mass; it then divides within its own periplast into eight small daughter-individuals or "merozoites," which may be with or without the rudiments of a locomotor apparatus; the merozoites then escape from the enveloping maternal periplast, and each one penetrates into a blood corpuscle, within which, in the general circulation, it develops into a full-sized trypanosome, which is finally set free from the corpuscle and swims in the blood plasma. According to Hartmann,[2] *Schizotrypanum cruzi* exhibits, in addition to the method of multiplication described by Chagas, a second method of multiplication, which takes place within greatly hypertrophied endothelial cells of the lungs; a final stage is figured, showing the cell containing some two dozen *Leishmania*-like bodies, each an oval corpuscle with

trophonucleus and kinetonucleus, but with no flagellum. The occurrence of "schizogony" in a trypanosome is regarded by Hartmann as a further proof of the supposed affinity between trypanosomes and malarial parasites—a doctrine which is one of the foremost tenets of the neo-Schaudinnian school.

Without entering further at present into these highly controversial matters, we content ourselves by remarking, first, that schizogony—that is to say, multiple fission not following upon sexual processes—is a method of multiplication that occurs throughout the protozoa, in every class, if not in every order, and therefore cannot by itself furnish any indication of genetic affinity; secondly, that the process of multiple fission within cells observed by us follows entirely the type of such multiplication as seen in trypanosomes; it is, in fact, essentially similar to the multiplication which *T. lewisi* is known to go through in the blood of the rat. In every case in which our observations permit us to make a positive statement, whether in stained preparations or in living material, we are able to assert that flagella are present during the whole process of multiplication; the large parent form retains its flagellum up to the spherical stage, and the daughter-individuals produced within the sphere are provided with flagella at a very early stage in their formation. It is, of course, possible that in some cases flagella may be absent; we have seen in the epithelial cells trypanosomes in the form of motionless oval bodies in which no flagella could be made out, and in one such case a body which had been perfectly quiescent for a long time was seen to wake up, as it were, and to begin moving about in the cell by means of a flagellum, though it would be impossible to say whether in this case the flagellum was a new formation, or had been present all along and had escaped notice in the cell. While, however, prepared to admit that the intracellular forms may in some cases be in a non-flagellated, resting, *Leishmania*-like stage, we venture to assert, from our observations as a whole, that the multiplication of *T. lewisi* in the stomach of the flea is typically the multiplication of a flagellate phase and perfectly different from the schizogony of a malarial parasite, which it resembles only in the broad features of multiple fission, as seen in protozoa generally. In the fact that it takes place within the periplast of the parent individual it resembles that described by Chagas in *Schizotrypanum*, but differs mainly in the following points: in *Schizotrypanum* an adult trypanosome of ordinary size, losing its flagellum, divides up to give rise to a number of minute daughter-individuals, which grow up into the adult form; in *Trypanosoma lewisi* an ordinary individual first grows to a relatively huge size, retaining its flagellum, and then divides into a number of full-sized and perfectly-formed trypanosomes.

References.

[1] *Memorias do Instituto Oswaldo Cruz*, vol. i (1909), pp. 176–180.
[2] *Archiv für Protistenkunde*, vol. xx (1910), pp. 361–363.

Observations on the Trypanosome of the Little Owl (Athene noctua), with Remarks on the other Protozoan Blood-parasites occurring in this Bird.[1]

By

E. A. Minchin, M.A., F.R.S.,

and

H. M. Woodcock, D.Sc.

With Plates 20 and 21.

The observations to be recorded in this and in a subsequent paper are the result of a study of the protozoan blood-parasites of the little owl, which was undertaken by us at Rovigno, Istria, the actual place where Schaudinn's celebrated investigation (**16**) on the same parasites was carried out. A re-investigation of these parasites has long been considered urgent, and stress has been laid by many people upon the importance of the work being done at Rovigno itself. The different views which have been taken with regard to the correctness or otherwise of Schaudinn's account are now well known, and it is scarcely necessary to recapitulate them here, more especially since they have been recently discussed by one of us in the first memoir of this series (**18**).

We were together at Rovigno for nearly three months, from about the middle of January, 1909. Unfortunately, we went much too early in the year. This was chiefly due to the fact that one of us (E. A. M.) was obliged to be in London from May onwards in order to deliver the University course of lectures, which had been previously arranged for

[1] "Studies on Avian Hæmoprotozoa," No. II. For No. I see 'Quart. Journ. Micros. Sci.,' vol. 55, p. 641.

that period. The other of us (H. M. W.) stayed on at Rovigno, continuing the work alone, until the beginning of July, when, after a stay of six months, he was also obliged to return on account of other work. There can be no doubt that if our visit to Rovigno could have been arranged for the six summer months, say from April to September, it would have been much more successful than it was. Still, we are able to bring forward certain definite observations, which may contribute towards the settlement of this difficult and much-debated question.

We take this opportunity of expressing our very grateful acknowledgments and thanks to the various people through whose courtesy and kindness we were enabled to undertake and prosecute this research; we were especially indebted to the late Dr. Hermes, the Director of the Zoological Station at Rovigno, for placing laboratory accommodation at our disposal, and to Dr. Krumbach, in charge of the Station, for his great assistance in endeavouring to obtain the owls.

The Birds and their Parasites.

At the outset we experienced a sad disillusionment in connection with the supply of owls. Although little owls were not scarce in the district, they were extremely difficult to procure. In the course of a month, in spite of all our efforts, we could only obtain five birds locally, which were brought in mostly in a wounded or dying condition after being shot. We tried several times to find haunts or nesting-places of the owls, but without any success. These difficulties in regard to the local birds did not affect us so much, however, as we feared at first would be the case, because (somewhat to our surprise), none of the above five birds showed signs of any hæmoprotozoan infection at all after thorough examination. After we had been at Rovigno a month we managed to obtain some owls from Vienna which were infected; and thenceforward we relied altogether upon dealers in Vienna and also in Breslau for our supply of birds. The only other local owl

examined, in fact, was a young fledgeling, brought into the laboratory about the middle of June. This little bird, which went by the name of "Piepsch," also had no parasites in its blood; it, too, was most probably quite free, but it became such a favourite that its sacrifice to science was not permitted. From our experiences with Rovigno owls we consider it quite likely that the birds in this neighbourhood were not infected with the protozoan blood-parasites specific to them; on the other hand, we never had more than two consecutive birds sent from Vienna or Breslau which were quite free from parasites.[1]

A great difficulty with which we had to contend was that the climate of Rovigno did not suit the inland owls, confined as they were in cages. As regularly as the periods occurred when the moisture-laden Sirocco prevailed, equally regularly did one or more of our precious owls die suddenly; and this danger of losing all at once an important bird continued to be a source of anxiety the whole time we were there. As a matter of fact the investigation would probably have been rendered much easier had we gone to Vienna to carry it out. There could not have been the slightest objection to choosing this district in preference to Rovigno, for in the course of our stay we gathered from Giovanni, one of the sailors attached to the laboratory, who was there in Schaudinn's time, that most of the owls which Schaudinn himself used came from Vienna.

Altogether we had eighteen owls sent from Vienna or Breslau, the birds being more readily obtained as the spring advanced. The accompanying table has been drawn up to show at a glance the number of birds infected, and the different parasites present in each case. (Tryp. = Trypanosoma, Halt. = Halteridium, Lz. = Leucocytozoon, and Prot. = Proteosoma.)

[1] Since our return home we have had a batch of four young owls sent from Geneva, all of which have proved entirely negative. This may be also an uninfected district.

Owls.			Parasites.			
No.	From	Date.	Tryp.	Halt.	Lz.	Prot.
6	Vienna	Jan. 26	—	—	—	—
7	„	Feb. 21	?	+	—	+
8	„	„ „	+	+	+	+
9	„	„ „	—	—	—	+
10	Breslau	March 17	—	—	—	—
11	„	„ „	—	+	+	+
12	„	„ „	—	—	—	—
13	Vienna	Apr. 16	+	+	+	+
14	„	„ „	+	+	+	—
15	„	„ 27	—	—	—	—
16	Breslau	May 8	—	—	—	—
17	Vienna	„ 23	—	—	+	+
18	„	„ „	—	—	+	—
19	„	„ 29	+	+	+	+
20	„	June 5	—	—	+	—
21	„	„ „	—	—	—	—
22	„	„ 12	—	—	+	—
23	„	„ „	+	+	+	+
Total, 18			5 (or 6?)	7	10	8

Analysis and General Remarks.

Twelve out of the eighteen birds were infected with one or more parasites, the remaining six being, so far as could be ascertained, quite negative. Of the different forms of parasite the Leucocytozoon occurred most frequently, being present in ten instances. Hence, more than 55 per cent. of the owls we obtained were infected with this parasite—a high ratio. Proteosoma was present in eight birds, i. e. in more than a third. Halteridium occurred in seven owls, and trypanosomes in either five or six. It will be noticed that no trypanosomes were found in any of the birds from Breslau, but only in those from the neighbourhood of Vienna.

With regard to the number of owls in which trypanosomes were present, we think it quite possible that owl 7, marked in the table with a query, may also have been infected, although trypanosomes were not actually found. Halteridia were also very scanty in owl 7. On the other hand, in owl 8 Halteridia, as well as most of the other parasites, were plentiful, and on

the death of this bird trypanosomes were readily found in its bone-marrow. When owl 7 died a few weeks later its bone-marrow was examined carefully, but no trypanosomes were seen and other parasites were also very scarce; consequently, only very few preparations were made from it since we regarded it as negative in respect to trypanosomes, and we had a number of good preparations from owl 8. The one smear of bone-marrow made from owl 7 had, unfortunately, very little on it, and was not much use; hence, and in view of our experience with owl 19, presently to be described, we cannot feel at all sure that trypanosomes were not present in owl 7, perhaps scanty in number.

Owl 11 had a good infection with Leucocytozoon, and Halteridium was also present; the latter form was excessively rare, however, only a few flagellating gametocytes being seen in the course of many living examinations, and the parasites being extremely difficult to find in permanent preparations. No trypanosomes were found in the bone-marrow of this bird. On the other hand, in both owls 13 and 14, the next birds obtained which had a good (or fairly good) infection with Halteridium, and in which Leucocytozoon also occurred, trypanosomes were found after death without much difficulty, as in the case of owl 8. Owl 19 also had a good infection with Halteridium, and when this bird died later on it was expected that trypanosomes would be found in it, as in the others; but in spite of very careful searching of fresh preparations for a period of two hours no indications whatever of trypanosomes were seen; a colleague, Dr. Reichenow, who was then working at Rovigno, also kindly examined a preparation without finding anything. Hence it was thought that in this case the Halteridium and the trypanosome of the owl had been definitely separated from one another, and that this was a case of infection with the former parasite only. However, good preparations from the organs of this bird were made for the sake of the Halteridium and Leucocytozoon, and in the systematic search of these undertaken since returning home, three small trypanosomes have been found in four

smears of the bone-marrow! The parasites were evidently extremely scanty; one of us, notwithstanding a fair amount of experience with Avian Trypanosomes, has never had another bird in which these parasites were so rare in the bone-marrow.

The incident of owl 19 emphasises the point that it is very difficult to be certain, from a living examination alone, that trypanosomes are absent from the bone-marrow. Small forms especially may be quite hidden by clumps of cells. The spreading out of a thin smear for a permanent preparation helps to set free and disclose the parasites.

The constant association of trypanosomes with one or more of the intra-cellular parasites, especially the Halteridium and Leucocytozoon, is very striking. In no case did we find trypanosomes in an owl which had not these two other forms also. Nevertheless, suggestive as this fact may be, we have been unable to obtain any evidence which would point to anything being concerned beyond coincident occurrence of these different types of parasite. Moreover, taking the other forms first, the coincidence is somewhat less marked. Thus, while five (possibly six) of the seven birds infected with Halteridium also had trypanosomes, in one certainly (No. 11) trypanosomes were not present; it is true that here the Halteridia were excessively scanty. Again, with regard to Leucocytozoon, this intra-cellular parasite occurred in five birds in which no trypanosomes could be found. In the case of three of these, Nos. 11, 17, and 22, in which the Leucocytozoon was either fairly frequent or else plentiful, good smears from the bone-marrow and also from the other organs (in the case of No. 22) have been well searched with negative result.

As will be gathered from the table, Proteosoma occurred indiscriminately, either alone or together with one or more of the other parasites; while in owl 14, which had the other three forms, Proteosoma was absent. An interesting point of difference may be mentioned between Proteosoma on the one hand and Halteridium and Leucocytozoon on the

other, the possible significance of which will be discussed further. In the early owls Proteosoma occurs chiefly in the form of schizonts; up to No. 11 inclusive, scarcely any gametocytes were found. In the later owls, on the other hand, the parasites were mostly in the gametocyte phase, schizonts being absent or rare. Halteridium and Leucocytozoon, on the contrary, occurred in the gametocyte phase in the earliest owls in which they were found—Nos. 7 and 8; many individuals were apparently ripe, since they "flagellated," or became rounded and free, according as they were of male or female sex, in the fresh coverslip preparations. Indeed, so far as these two intra-cellular parasites are concerned, we may take this opportunity of saying that, as regards endogenous multiplication or schizogony, we have been quite unable up to the present to obtain any evidence of such a process, and this in spite of much searching, since our return home, of what we considered very promising material.

Owl 23, for instance, had the strongest Halteridial infection that either of us has ever observed; nearly every red blood-corpuscle was infected with three or four—sometimes more—of these parasites, the majority of them being small forms. Whence this host of parasites originated remains a mystery; neither smears nor sections of lung and spleen have as yet shown anything different from the condition found in the general circulation. And the same result has attended the searching of preparations of the organs of earlier birds, where the Halteridia were not so abundant in the blood. The point remains for future investigation.

Habitat.

Turning our attention now more particularly to the trypanosomes, it may be remarked in the first place that, so far as our observations have gone, the trypanosome of the little owl shows a general correspondence with that occurring in the chaffinch and redpoll, described in the first memoir. This is

the case both as regards the habitat of the trypanosomes and their behaviour in the bird at different seasons, and also in regard to the chief morphological types found.

In the winter and spring months the bone-marrow is the chief seat of the parasites; indeed, for all that we could learn to the contrary, they may be restricted to this situation. The blood of owls 8, 13 and 14 was examined living on many occasions, both in the day-time and at night, and numerous stained preparations of the peripheral blood and the internal organs (lungs, spleen, etc.) have been searched, without in any case coming upon a trypanosome.

Nevertheless, from the experiences of one of us in reference to this point, in working on other birds, we should not like to say certainly that the parasites do not occur at all in the general circulation at this period.[1] Most unfortunately the indispensable test, that, namely, of taking cultures from the blood, was quite a failure in its application in this case. Several culture-tubes were inoculated from the peripheral blood of the above owls, which remained uncontaminated, or practically so, by bacteria, but they were also sterile as regards trypanosomes. The disappointment was, however, that in tubes inoculated from the bone-marrow of these birds, in which trypanosomes were known to be present, no cultural forms developed. In no instance, in fact, did we succeed in obtaining a culture of the owl-trypanosomes, a very different result from the usual experiences with the culture-method. As Rosenbusch (**15**) has obtained cultures of the parasites—i.e., as will be shown later, of the trypanosomes—of the little owl, we can only surmise that the fault in our case lay with the tubes used.[2]

[1] Mayer, however, has noted (**12**) the occurrence of the parasites (in the large, massive form, c.f. below) in the general circulation in the early part of the year (January) in the case of the trypanosome of Syrnium aluco.

[2] We had no facilities for making culture-tubes at Rovigno, and had to rely upon some taken with us; many of them were rather old by the time they were required; others had to be boiled up and fresh salt-

On the other hand, in the summer, for instance in owl 23, the trypanosomes certainly occurred in the general circulation. They were seen for the first time in blood taken from the living bird on June 15th–16th, in the night-time, after being five months at Rovigno! This fact is mentioned just by way of illustrating the difficulties attending the path of the would-be researcher on these Avian parasites. The trypanosomes were found also in subsequent examination of the bird; and in permanent preparations made they are not infrequent, averaging four to six on a good-sized film. Even at this time, however, the parasites are somewhat more numerous in the bone-marrow, as we have learnt from our preparations made after the death of this bird (which took place on June 29th), while they appear to be extremely scanty in the smears made from the internal organs (lung, spleen, etc.).

Living Observations on the Parasites.

With a view of ascertaining whether we could find anything which pointed to the actual passage or transformation of the trypanosomes into Halteridia or Leucocytozoa, or vice-versâ, careful living observations were made on several occasions, usually in the night-time.

A. Trypanosomes.—The following notes were made at the time of the examination of the bone-marrow of owl 8 after its death (February 26th, 1909), in which, of all our infected birds, trypanosomes were most numerous to this situation. The parasites observed were of three distinct kinds: (1) Slender, active forms, of medium size, with a finely drawn-out aflagellar extremity. In two instances such forms were seen attached by this end to a cell of leucocytic character; the trypanosomes were lashing about in every

citrate added. Our tubes were, therefore, not very satisfactory; but we were not prepared for the entire failure of the trypanosomes to appear in them, considering the readiness of these Avian parasites, as a rule, to develop in cultures.

direction, apparently unable to free themselves, and were dragging the cell here and there. (2) Large, stout, sluggish forms, with a very long, tapering aflagellar end, and a much pleated undulating membrane; the free flagellum was short. These individuals travelled very slowly, scarcely moving from one place; they often seemed to get wedged in among clumps of cells. The membrane, however, was in a continual state of rippling, and an interesting point was noticed in connection with this, namely the reversal of the movement. The waves ran usually from the region of the kinetonucleus towards the flagellum, and the movement in this sense was continued for the longer period; but it was frequently seen to change, when the waves ran from the flagellar end towards the kinetonucleus, for a short time. All of a sudden, and at irregular intervals, the movement would change again and be once more in the original direction. (3) The third type of parasite seen was quite a small form, not much longer than a blood-corpuscle; these individuals were somewhat stumpy in appearance, with the aflagellar end abruptly pointed. These forms were not noticed at first; they were probably overlooked through being hidden by or among clumps of cells, out of which they managed later to worm their way. They had a wriggling movement, but did not seem to travel much. These small trypanosomes appeared to have a predilection for attaching themselves by the tip of the flagellum to the surface of the glass, either to the slide or the cover-slip.

On examining a preparation the following morning (some ten hours later), one of the small forms was observed slowly writhing about, quite free from any corpuscles, but with the tip of its flagellum firmly attached to the slide by a distinct dot or granule. The trypanosome, by its movements, pushed and pulled its body from and to the spot where it was attached, without, however, becoming free. The membrane undulated slowly; its flagellar border appeared distinctly to be wound spirally round the body of the parasite. The body itself seemed somewhat stiff, and only changed its curves slightly and slowly. Another trypanosome, probably belonging

to the first type mentioned, was also noticed at this time; it was recognised by its movement, but as it was more or less surrounded by a clump of corpuscles, its characters, with the exception of its approximate size, could not be made out. Looked at again later (about noon) the small trypanosome was found to have detached itself, and was wriggling feebly, loose, and waving its flagellum about slowly; it appeared moribund. At 2 p.m. it was nearly dead, only the tip of its flagellum now moving feebly.

The same three types of individual were also found in the bone-marrow of later owls (Nos. 13 and 14); and here again, in the latter bird, certain trypanosomes were seen to be distinctly attached. A medium-sized, slender form (belonging to the first category) was attached to a corpuscle by its flagellum, by means of which it was waving the corpuscle about vigorously. Further, a small trypanosome (type 3) was seen unmistakeably attached by its flagellum to a leucocytic cell; and another small individual also appeared attached, but this could not be ascertained with certainty owing to the parasite being partially hidden. These observations were also made in the evening, about 8 p.m.; and the above two individuals were watched at intervals until midnight without any change being noticed. Next morning the slender form could not be found again; while the small individual was quite motionless and seemed to be dead.

From the above notes the following definite and rather interesting points may be emphasised. The slender, medium-sized parasites, and also the small, stumpy forms, may be found attached to a cell, either by the flagellum or by the aflagellar end. On the other hand, no individual belonging to the large, sluggish type (of the second category) was ever observed attached.

In owl 23 quite a different state of affairs was met with from that obtaining in the earlier owls. The trypanosomes in this bird were in what we propose to refer to as the "summer condition"—both as regards the habitat of the parasites and the type of form found. This condition is to be

recognised, we think, as constituting a perfectly definite phase in the life-history of these Avian trypanosomes. As above mentioned, the parasites were observed living in the peripheral blood on two occasions, in the night-time; only very few trypanosomes were noticed altogether in several cover-slip preparations. They belonged to one type, and were fairly small and stumpy, spindle-like or rather broad; as will be seen later, these forms are not quite comparable to those of the third category above described. All the trypanosomes observed living in this owl, whether in the blood or (later) in the bone-marrow, were free; none showed any inclination to become attached to any cell.

To sum up: In spite of many and long-continued observations we never succeeded in seeing any form of the trypanosome of the little owl either penetrate into, or become completely attached to a cell, and lose concurrently its locomotor organs; and this notwithstanding that both Halteridia and Leucocytozoa were present in all cases in the same birds. The only manner in which we observed the trypanosomes to be associated with cell-elements was that of their attachment by one extremity—which might be, apparently, either end indifferently; and we never saw this attachment develop into any closer connection (cf. however, below, p. 165).

B. Leucocytozoon.—We do not intend in this paper to discuss the minute structure of these parasites, but only to note certain features observed, and more especially those bearing upon the question of the actual connection of these intra-cellular parasites with the trypanosomes. Owl 11 had a good infection of Leucocytozoon and was the most suitable of the earlier birds for studying this form. The parasites were all in the gametocyte-phase, most of them being nearly full-grown in size. In nearly all the host-cell had acquired the characteristic spindle-like appearance, with the cytoplasm drawn out in two long, tapering processes. The degree of plumpness of the parasites varied slightly; some were more slender, others rather stouter; but apart from

this, there was strikingly little difference to be noticed among the numerous parasites observed. We certainly never saw anything resembling the curious appearances and behaviour of the parasites described by Dutton, Todd and Tobey (**3**) in another species of Leucocytozoon; and in this respect our observations concur with those of Wenyon (**17**).

Many individuals were watched very carefully for signs of movement, but in no instance did we see any active movement or change of form, either of the ovoid, more internal (endoplasmic) part of the parasite or of the tapering extremities of the spindle. The latter, probably consisting at any rate in the narrower portions only of the cytoplasm of the host-cell, were sometimes seen to bend slightly to and fro, quite passively, this motion being caused, doubtless, by little currents in the blood-plasma in the immediate neighbourhood. We never observed any amœboid movement or anything comparable to the waves of constriction described by Wenyon in the case of L. neavei; neither did Mathis and Léger (**8** and **10**) in their form from the fowl. Of course, in referring to this absence of movement in the parasites, we are not taking into account the preliminary efforts of ripe gametocytes either to rupture the host-cell or to form microgametes. It is possible that the observations made by Wenyon may refer to such ripe gametocytes which were endeavouring to free themselves from the leucocytes; though it is true we always found this process to occur very rapidly in L. ziemanni. The reason why we have laid stress on the entire absence of active movement in the ordinary spindle-shaped individuals—which was always the case in every bird in which we studied them—is to emphasise the fact that we never saw any indication whatever of the development of locomotor organellæ, or of any active trypaniform phase. Further, we have never once found, either in living preparations or in the permanent smears, any young or intermediate-sized individuals or forms other than the ripe gametocytes, free in the blood-plasma, however numerous the parasites were.

An interesting feature which may be pointed out was the variation we noticed, both in the number of gametocytes present in the drops of blood examined and in the apparent ripeness at different times. Mathis and Léger (9) have remarked on the occurrence of what they describe as a periodicity of the gametocytes in the circulating blood, which they noticed on two occasions in Leucocytozoon caulleryi, of fowls in Tonkin. The parasites would be observed for a certain number of days (four to seven) in varying frequency, and then would apparently disappear from the blood and not be seen again, often for some weeks. In the cases instanced there appeared to be no regularity either about the length of time during which the parasites were not observed, or about the period, usually a few days, when they were present.

We never observed in any of our owls infected with Leucocytozoon a complete disappearance of this parasite from the blood. The following notes illustrate the variation to which we have just alluded in owl No. 11.

March 18th, morning after arrival: Leucocytozoon present; parasites frequent.

March 19th–20th, midnight: Parasites distinctly more numerous, approaching abundance. Many rounded-off forms (i.e. ripe females, which had ruptured the host-cell) seen. Probably ripe male individuals were also present, though no flagellating forms were actually seen.

March 23rd, morning: At first no signs of Leucocytozoon, but after some searching the parasites were found. They were infrequent to scanty, only five or six individuals, one of which was rounded-off, being noticed in four cover-slip preparations.

March 23rd–24th, midnight: Parasites numerous. In a cover-slip preparation of pure blood only spindle-shaped, intra-cellular forms were seen; but in a preparation to which a drop of salt-citrate solution had been added, many liberated females and some flagellating male gametocytes were found.

March 27th–28th, midnight: Leucocytozoon not at all frequent, rather scanty in fact, requiring considerable searching. Parasites distinctly in batches. In one drop none were seen after much searching, while in another four or five intra-cellular forms were come across one after another fairly quickly. In a drop diluted with salt-citrate solution two ripe females and also two flagellating males were found.

March 30th, morning: Leucocytozoon numerous. Many rounded-off females observed, but only one flagellating male.

April 3rd, afternoon: Owl 11 died. Leucocytozoon numerous, both in blood and internal organs. No rounded-off or flagellating individuals were observed, however, whether in preparations to which salt-citrate had been added or in those to which it had not been.

In several of our other owls in which Leucocytozoon was present (e.g. Nos. 14, 17 and 19), the parasites were infrequent or scarce whenever the birds were examined (this being the case even in the internal organs after death), and consequently no such variation in number was observed. In owl 22, however, which had a very good infection with Leucocytozoon, the above phenomenon was again noticed.

June 13th, morning after arrival: Leucocytozoon numerous; many flagellating as well as rounded-off individuals seen.

June 21st–22nd, midnight: Parasites not nearly so numerous, only fairly frequent, and no flagellating or rounded-off individuals were observed.

June 22nd, afternoon: Owl 22 died. Both in the heart-blood and in the bone-marrow Leucocytozoon was fairly numerous, distinctly more frequent than in the peripheral blood the night before, but again no ripe forms were seen—either rounded-off or flagellating.

Of our birds infected with Leucocytozoon, the one which we had under observation for the longest period was No. 14, which was first examined on April 20th and lived till May

20th—just a month. Leucocytozoon was very scarce in this bird. It was not found in the living examination on April 20th; but on April 23rd a single individual was noticed in two cover-slip preparations. Examined again on May 9th (morning) and also on May 12th–13th (midnight) no Leucocytozoa could be seen in the living drops. But a very few individuals have since been found on searching permanent smears made on these occasions. Hence the parasites were present in the general circulation, though so scanty that their presence could not be demonstrated in the routine examination of several living drops on both occasions. Further, in the smears made from the heart-blood of the dead bird on May 20th Leucocytozoon is also present, though very scanty.

From the above observations we think, therefore, it is practically certain that in none of our owls infected with Leucocytozoon was this parasite at any time really absent from the general circulation.

The variation in number and ripeness of the gametocytes, and their occurrence at times in distinct batches, is most probably the result of some antecedent process of schizogony, by which the sexual forms have originated. A schizogonic mode of multiplication has been briefly described by Fantham (4) in L. lovati of the grouse; and a similar phase occurs in all likelihood in other Leucocytozoa. At a particular moment, we may suppose, a number of young (potential) gametocytes are liberated by the breakdown of the host-cell in some internal organ in which they have been developed. They penetrate, probably as soon as possible, into the new leucocytic host-cells in which they will grow and mature, and so pass into the blood-current, in which they are passively borne along. As it is quite probable that different host-cells (or groups of host-cells) containing the products of schizogony become ruptured at different times, we should have, in that case, clumps or batches of gametocytes of slightly different age and ripeness in different small quantities or volumes of blood.

The only other explanation of our observations—on owl 11, for instance—would be that the great majority of the numerous gametocytes observed on March 19th–20th had died off by the 23rd, and that those seen again on March 23rd–24th were an entirely fresh lot, which had been very rapidly developed as the result of a quite recent schizogony. In the first place it is most unlikely that the gametocytes would die off in such numbers as soon as, or even before, they were quite ripe, especially at the beginning of the season—such a course would be very expensive and most unusual for a parasite. Secondly, if schizogony had been going on recently to produce the numerous forms seen in the blood March 23rd–24th, and again, subsequently, to give rise to those present on March 30th, we should certainly have expected a proportion of these forms to be small to intermediate in size, which is not the case; and, moreover, we ought undoubtedly to have found some indications of the actual schizogonic process in our permanent preparations of the internal organs made on April 3rd, when the bird died. As a matter of fact in all our infected owls the schizogonic process seemed to be over and done with; altogether we have only come across extremely few really small (young) gametocytes. Hence, the first explanation we have offered appears by far the most reasonable.

It is interesting now to compare with our experiences those of Mathis and Léger in the case of the species parasitic in Tonkin fowls already alluded to. It is important to note that the periodicity described (occurrence for some days, alternating with apparently complete disappearance for a period of two or three weeks or more) was observed only in L. caulleryi; in L. sabrazesi from the same bird, which was made a distinct species on account of the gametocytes always having the spindle-like shape, this disappearance was not found. In a previous memoir (18) it has been remarked by one of us that those two species are most probably only different phases of one and the same parasite. The L. sabrazesi phase probably corresponds in a general way

to the condition in which we found L. ziemanni in some of our owls. The L. caullery*i* phase, on the other hand, appears to represent an older condition of the parasites—probably a much older infection—in which the gametocytes are quite mature, it may be, over-ripe. In such a case one may suppose that many of those which do not succeed in passing into the alternate host at length die off. It is not unlikely, we suggest tentatively, that others are able to undergo some parthenogenetic development and give rise later to a fresh succession of gametocytes in the blood. In this manner the reappearance of the parasites after an interval could be readily explained.

We may conclude our remarks on Leucocytozoon ziemanni by giving extracts from our notes relating to one or two interesting observations on the gametes, which we were fortunate enough to find in living preparations. The male gametes were first detected by the movements of the corpuscles which they caused. They appeared as very slender, spirochæte-like bodies, exceedingly active, performing twisting movements and travelling at a fair pace; they were rather longer than a red corpuscle and capable of jerking the corpuscles about. Three or four of these delicate elements were seen in an area rather larger than a field of the microscope. In another field an active male gamete was found and also a rounded-off female individual; the latter was quite spherical and had ruptured its host-cell, the remains of which, together with the nucleus, were still attached to one side of the parasite. The male gamete was at first some little distance from the female element; it travelled fairly fast, and in keeping it in view the female individual was sometimes quite out of the field (of the oil-immersion lens), and then would be brought in again, i.e. the microgamete was sometimes nearer to, at other times farther from, the female. Once the male was seen to travel in a straight line rapidly towards the female till it nearly reached it; but then it turned off to one side again. After moving about a little longer, however, the microgamete at

length approached the female gamete, and after some gyrations, which caused the latter to be jerked about as well as the corpuscles in the vicinity, it was seen to be definitely attached to the female by one extremity. The male element continued to lash its body and jerk the female about, but after a time these movements became feebler and the male seemed to be contracting. At this moment the female was suddenly violently jerked, and after that no further movements were seen on the part of the parasite. The female individual was now examined very carefully but no signs of any little body attached to it could be made out; the male gamete seemed to have been absorbed. At this period no definite nucleus could be distinguished in the female; one part of the body was clearer and free from pronounced granules, but no sharply contoured nucleus could be made out. After watching the parasite for some time it was noticed that a red corpuscle, flowing slowly past it, was deflected slightly from its course, and it was seen that there was then a small body attached to the parasite at this point; this little body was spherical, of a definite contour, and contained a few granules; it appeared like a minute cell or nucleus. Shortly after this minute body had been found and when our attention was again turned on the parasite proper, it was observed that its nucleus was now quite distinct. It could be seen as a slightly oval clear space, with sharp and definite contour, and had near one end a dull spot, quite different in appearance from the much darker grains in the cytoplasm. The diameter of the nucleus was about double that of the little body attached to the female gamete. The parasite was watched for some time longer, but the only change observed was that the attached body seemed to become rather contracted and shrivelled, as if degenerating. No indications of any other change or development in the parasite itself could be noticed. When seen again in the morning (of the same day) the Leucocytozoon had become hyaline in appearance and seemed to be dead; there was no sign of the little attached body.

This was the only occasion on which we were able to see the fertilisation of a female gamete of Leucocytozoon, but several times we observed the male gametocyte in the act of flagellating, i.e. of developing the microgametes. This process occurred more readily in coverslip preparations to which a drop of salt-citrate solution had been added than in those put up of pure blood alone. When the microgametocyte ruptured its host-cell its body protoplasm was usually more or less segmented or divided up into two or three lobes or portions from which the male elements were given off, just as was described and figured by Schaudinn (loc. cit.). The number of microgametes formed appears to be variable. Schaudinn gives the number as eight; on the other hand, Laveran (**7**) figures four as arising, also in this species of Leucocytozoon ("Hæmamœba" ziemanni). In one case we saw three quite distinctly, and there may have been a fourth, but we could not be certain; in another instance, where the body of the parasite (freed from the host-cell) had been constricted into two masses, only one male element was seen to be formed. It is not improbable that in the citrated drop some gametocytes may be stimulated into attempting to develop microgametes before they are really quite mature enough to do so in a completely normal manner. Thus in the last instance given, the solitary microgamete, at first flagellum-like and active, appeared unable to liberate itself from the protoplasmic mass, and after five or six minutes its wrigglings became less active and more spasmodic, and finally it became much contracted and pear-shaped and ceased to move. In other cases, again, the microgametocyte did not succeed in rupturing the enclosing envelope of the host-cell, and the microgametes were developed inside the skin or capsule, as it were, of the leucocyte, from which they were unable to get free. In one instance several male elements (there may have been as many as eight) were seen thus imprisoned; they were in two bunches, directed towards the spindle-like ends of the host-cell, and were lashing themselves about vigorously in the

endeavour to become free. This movement went on for about three quarters of an hour, but with no success.

The free microgametes themselves were, as already mentioned, very slender thread-like bodies; they were, if anything, rather longer than those of Halteridium. The gametes, living, were observed very closely, but no signs of any undulating membrane could be made out; nor could any more active, whip-like part of the body, corresponding to a free flagellum, be distinguished. Unfortunately, we have not been able to find any microgametes in our permanent preparations; Wenyon, however, has figured (17) the male elements of L. neavei, from a stained preparation, and these also appear simply as threads. It is most likely, we think, that the minute structure of the microgametes of Leucocytozoon is very similar to that of the corresponding elements in Halteridium; in the latter parasite, the male gamete, as has been described by one of us (loc. cit.), consists of a delicate cytoplasmic thread, containing two or three chromatic masses of varying size and having a distinct centrosomic granule at one extremity (cf. also below).

c. Halteridium.—Three of our owls had a good Halteridial infection, Nos. 13, 19, and 23; the parasites were plentiful in the first, abundant in No. 19, and simply swarming in No. 23. It may be remarked here that the terms "numerous," "abundant," and so on, as we have used them, do not mean the same thing, as regards the actual number of the parasites present in the case of Halteridium and Leucocytozoon respectively; this will be understood when the different habitat of the two forms and the relative proportion of red blood-cells to small mononuclear leucocytes is borne in mind. For instance, we may consider Leucocytozoon to be numerous when two or three individuals on an average can be seen in a single field of a fresh cover-slip preparation, working with a dry lens (obj. D or 4 mm. apochromatic); but we should not regard Halteridium as abundant in an infected bird unless on an average at least one or two individuals occurred in a single field of a stained smear,

using an oil-immersion lens. Such an infection was present in owl 19. And in owl 23 nearly every red blood-corpuscle is infected; very few uninfected red cells can be found in the permanent preparations; there are nearly always two or three parasites in a single host-cell, and frequently their number is four to six, when they are mostly small or quite minute forms.

No pronounced variation in the number of the Halteridial parasites present on different occasions of examination was observed, contrary to what was so markedly the case in Leucocytozoon. It was observed several times, however, that there was distinct, often considerable variation in the number of individuals which were ripe enough to flagellate or become rounded off. Thus, at some examinations, by the time a drop of pure blood could be mounted and put under the microscope numerous male gametocytes would be seen actively liberating free microgametes, rounded-off female forms also of course being present; at other times scarcely any such, or none at all, would be found. Another noticeable point of difference from Leucocytozoon was that not only adult or nearly adult individuals, but also young or small forms and forms of intermediate size, were nearly always present at the same time in the blood.[1]

On the whole, comparing the results of our observations on Halteridium and Leucocytozoon, we think the following conclusions are suggested. The schizogonic process in the former parasite must be on a considerably larger scale than it is in the latter, even if we suppose that there is a stronger original infection.[2] Further, it is probable that the schizogony in Halteridium may be of longer duration, i. e. that it may continue to go on for a longer period than is the case in the Leucocytozoon; this seems to us to be indi-

[1] This agrees with the condition which was found in the case of H. fringillæ, in the chaffinch.

[2] If schizogony in the parasite of the little owl is similar to the process described by Aragao (1) in the Halteridium of the pigeon, it is indeed on a lavish scale.

cated by the fact that in our early owls as well as in our later ones quite small forms, which cannot have been long set free from the parent schizont, occur as well as others of medium size in the red blood-cells; also because the later the bird, the greater the number, as a rule, of Halteridia present (owls 8, 13, 19 and 23 form a regular series in this respect).

In the birds in which Halteridium was fairly numerous or abundant (Nos. 13, 19 and 23) parasites were seen in the living cover-slip preparations which were quite free from a blood-corpuscle, although they were not rounded-off or flagellating individuals. Only a few of these free Halteridia were noticed in owl 13, they were less scanty in No. 19, while in No. 23 they were quite common. In the last case these free forms varied considerably in size, from small individuals up to forms of intermediate size or larger. These free forms appeared, so far as could be seen, perfectly similar to those in the corpuscles. It is important to note that they were quite motionless and were not observed to undergo any change. Particular individuals, fairly large ones, were watched for two or three hours at intervals, and at the end of that period had not altered at all. Not the least indication was seen of the development of any of these free Halteridia into a trypaniform condition. In the living blood from owl 23, examined at night, one or two trypanosomes were found; these were distinctly larger than the free Halteridia. In spite of much searching, no parasites were seen to become actually liberated from the corpuscles. It is certain, however, that most, if not all, of these free individuals had been parasitic in a red cell, for the great majority contain pigment-grains.

The same state of affairs observed in the living preparations from these three owls is found in the permanent smears made from them respectively. Here and there in the preparations from owl 23, where, as already mentioned, several parasites often occur in a single corpuscle, three or four free individuals are found grouped around or else close to the isolated nucleus of a red cell; in such cases one may assume

that the cell has been disorganised artificially in making the film. Most of the free forms are more or less uniformly dispersed, however, and have no broken-down remains of a red cell in their vicinity. This fact, and, of course, the occurrence of these forms in the fresh preparations, makes it certain that their liberation is not due merely to manipulation of the blood. These free forms are undoubtedly present in the blood in the bird, scanty or numerous as the case may be. We are left somewhat uncertain, however, whether the process of liberation is an active or passive one on the part of the parasites, i. e. whether the Halteridia leave the red blood-cells of their own accord, by breaking out or away from them, or whether they are simply set free by the rupture of a used-up corpuscle, which has probably contained several parasites. Schaudinn maintained, of course, that the intra-cellular Halteridia regularly become active and trypaniform, and voluntarily leave the corpuscles. We should have seen this process in owls 19 and 23 if it occurs. On the whole, we are much more inclined to think the latter explanation we have suggested is the true one. In this connection it is important and interesting to note that there is markedly little or no hypertrophy and enlargement of the red blood-corpuscles by the Halteridia, even when there are several individuals in one host-cell; this contrasts strikingly with the great enlargement and stretching which the red cells may undergo when infected with Hæmogregarines or Hæmocystidium, for example. Hence it seems to us most likely that in an abundant infection, when there are three or more Halteridia in a corpuscle, their growth and increase in size renders it only a question of time before the corpuscle is ruptured, thus passively liberating the enclosed parasites. We should say it is very doubtful whether such free forms become again intra-cellular.

Mayer, in his account of the parasites of another owl, Syrnium aluco, also admits that he sought in vain for active trypaniform phases (trypanosome-forms) of the Halteridium.

Before leaving this question we should like to put forward

a suggestion, which is, however, nothing more than a suggestion. We think it is not at all impossible that there may be some intra-cellular phase of trypanosome, perhaps even occurring in a red blood-corpuscle, still to be definitely ascertained. As was discussed in the preceding memoir with reference to the recent work of Chagas (**2**) and Hartmann (**5**), it is reasonable to suppose that a schizogonous process of multiplication occurs in the life-history of these Avian trypanosomes, by which the small forms are developed from large, massive individuals. And it is quite possible that the immediate products of schizogony, which may be assumed to be small elements, pass for a time into the red cells before being liberated as small, active trypanosomes; such intra-cellular phases would most likely be found, we think, to be quite independent of the Halteridia, and might not possess pigment. In the scheme drawn up on p. 176, showing the relation between the different phases of the trypanosome, as found in the owl, we have indicated the position which this hypothetical intracellular form would occupy.

In this connection reference may perhaps be made to a remarkable passage in Zupitza's account of Avian trypanosomes (**19**). Zupitza states that in blood taken from a wounded "Haarvogel" ("Bülbül"), in which both trypanosomes and Halteridia were present, he observed two or three red blood-corpuscles which were undergoing a strange spontaneous movement, turning over now in one direction, now in another. Further wave-like swellings of the membrane or envelope of the corpuscle were noticed. According to Zupitza careful examination of these phenomena showed that they were due to a small, slender trypanosome inside the corpuscle, which coursed rapidly through the cytoplasm, around the nucleus, first in one sense and then in the opposite one. Apparently the trypanosome was trying to bore a way out through the envelope of the cell, but so long as Zupitza was able to watch it it was unsuccessful. We shall have to criticise Zupitza's paper in many respects, but we feel quite unable to offer any comment upon this wonderful description;

all we can do is to direct attention to it. Unfortunately, in the course of very numerous observations on birds infected with trypanosomes, neither of us has ever seen anything in the least corresponding.

Morphology of the Trypanosomes as seen in the Permanent Preparations.

All our permanent preparations are in the form of smears. Both the two chief methods of preparing films were employed, namely: (1) fixation by osmic acid vapour, followed by absolute alcohol, and then staining by Giemsa; and (2) wet fixation of smears made on cover-slips, either by sublimate-acetic mixture or by Schaudinn's fluid, and then staining by iron-hæmatoxylin. The details of our use of both these methods have already been described (see Minchin [**13**] and Minchin and Woodcock [**14**]), so they need not be repeated here. The drawings are all magnified 2000 times, so that a particular individual can be at once compared with any other.

The type or types of form presented by the parasites varied in different birds. In the early owls infected with trypanosomes (Nos. 8 and 13), three readily distinguishable types occurred, as has been indicated above in our account of the observations made on the living, active parasites. These three types may be distinguished as small, medium, and large respectively. While each type has sharply defined characteristics, and at the first glance might be regarded as representing a parasite quite distinct from both the others, nevertheless, as will be seen below, forms occur which are transitional in character from one type to the other, and there can be no doubt, we think, that all three are only different phases of one and the same trypanosome.

The small type of individual (figs. 1, 2, 14, 32–35) is spindle-like or fusiform in shape. The aflagellar extremity of the body is abrupt and conical, the kinetonucleus being situated close to the end. The undulating membrane is relatively quite well developed, and the free flagellum is fairly long

Using the standards of measurement previously adopted (loc. cit.), the total length of these forms, as they appear on smears made by the first (Romanowsky) method (figs. 1–3, 14), is from 26 to 28 μ, and the greatest width 3 to 4 μ, while the length of the free flagellum is usually about 8 to $9\frac{1}{2}$ μ. The trophonucleus is situated about the middle of the body, or at most is only slightly nearer to the aflagellar end than to the other.

Comparing these forms as they are seen in wet films (i.e. films made by the second method), the length of the parasite appears uniformly somewhat less, averaging about 25 μ. There is no doubt that the body of the trypanosome is always rather contracted or shrunk by the wet method of preparation. This can be clearly seen to be the case by comparing figs. 32–35 of these small parasites on wet films with the figures of individuals of the same type on Giemsa smears. It will be noticed also that, in the former case, the flagellar border of the parasite frequently appears more crinkled and angular-looking than in the individual on "dry" smears; this being the result of a shortening of the body cytoplasm to a greater extent than the flagellar border (though the latter, and of course the free flagellum, is also somewhat contracted).

The next type of the parasite, the medium-sized form (figs. 7, 8, 20, and 21), is distinguished by its long, finely tapering aflagellar region, and also by the conspicuous undulating membrane, the folds of which are broad and high. The folds, it may be noted, frequently show the delicate endoplasmic intrusion, as described in the first memoir. The aflagellar end may be very narrow and attenuated (figs. 7 and 20). The free flagellum is relatively long. The entire length of these forms, in the above typical instances, is from 44–47 μ, the greatest width varies from 5 to $5\frac{1}{2}$ μ, the length of the free flagellum is 11 to 13 μ, while the aflagellar prolongation of the body, measured from the kinetonucleus, is usually 5 to $6\frac{1}{2}$ μ, but in the individual of fig. 7 it is as much as 9 μ. In this type the trophonucleus is always in the aflagellar half of the the body, i.e. it is nearer to the kinetonucleus than to the

point where the cytoplasm ends and the flagellum becomes free.

The cytoplasm of both the types of form just described is coloured either pale blue or a faint lilac in Giemsa smears. Usually it appears fairly homogeneous in character (figs. 20, 21), but now and then it contains granules, more or fewer, which stain dark red (fig. 8).

The third distinct variety of form shown by the trypanosome is very large, and also differs markedly from the other two types in the appearance of the cytoplasm (figs. 9–11, 22, 24). This is very dense, and in Giemsa-stained smears is coloured a deep and intense blue, which may be slightly tinged with purple or lilac. The aflagellar region of the body is prolonged for some distance beyond the kinetonucleus. It usually tapers to a fine extremity, but it is never so narrow and attenuated as in the medium-sized parasites, because in these large forms the body is much broader at the level of the kinetonucleus than in the latter. The undulating membrane may be very prominent, especially towards the flagellar end of the body. The dimensions of some of the largest of these forms (figs. 10, 22 and 24) are as follows: Total length 54–60 μ, greatest width $5\frac{1}{4}$–6 μ, length of aflagellar prolongation 12–15 μ, and length of free flagellum $8\frac{1}{2}$ μ, the last named being comparatively short. A rather shorter but broader individual (fig. 11) measures 50 μ in length, $6\frac{1}{2}$ μ in width, while the aflagellar part is 9 μ long, and the flagellum again $8\frac{1}{2}$ μ.

In wet films, stained by iron-hæmatoxylin, this type is readily distinguishable (figs. 43 and 44), both by its general shape, which agrees quite well with appearance of similar parasites on the other smears (allowing for a uniform shrinkage in size), and also by the staining reaction of the cytoplasm, which is stained dark grey, a much deeper tint than in the case of the small or medium-sized individuals, whose cytoplasm is quite pale or else only slightly stained. The largest examples of this type, as seen in wet films, measure 44 μ in length, $4\frac{1}{2}$ μ in breadth, the aflagellar region is 7 μ long, and

the free flagellum 7–10 μ; it may be, of course, that these particular examples are not really quite as large as that, for instance, of fig. 10, making allowance for some contraction.

All the individuals of this large type which we have found in wet films have the same general shape and appearance—that of a long, rather thick spindle with finely tapering extremities; and this form agrees closely with the appearance of these individuals as they were observed alive. Hence we feel sure that on Giemsa-stained smears, those individuals which closely resemble the above-described parasites on wet films can be correctly regarded as having retained the typical and normal appearance; and it is from such standard examples that we have taken the measurements given above. This is an important point to note, because these large parasites are difficult to obtain well fixed and stained on a Giemsa smear. Not uncommonly they are found of the weirdest shape and appearance; we have not the least doubt that such individuals have been deformed and distorted in making the preparation. Such parasites are generally very much flattened out, while the aflagellar end is blunt and broad and has quite lost its true shape; sometimes the whole trypanosome may appear nearly rectangular. It is quite useless to figure such individuals; Zupitza (**19**) has given (fig. 49, pl. 5), an excellent illustration of how far removed from its true shape one of these large forms (which he regards as "T. ziemanni") may appear on a dry, Giemsa-stained smear. Unfortunately, from his description Zupitza apparently quite fails to realise that the individual he figures is hopelessly flattened out and distorted.

The question of the flattening-out of these large forms, which is, of course, liable to occur on a "dry" film, has an important bearing, we think, on another point. In the memoir on the trypanosome of the chaffinch and redpoll, the corresponding large blue forms there described showed in most cases a characteristic structural peculiarity of the cytoplasm, namely its tendency to show an arrangement into longitudinal bands, dark and light alternating, the former

apparently composed of coarser, more closely packed granules. It was stated then that these bands were not to be regarded as actually representing myonemes. Now, in the case of the large blue trypanosomes from the little owl, most of the well-fixed individuals, i.e. those most closely approximating in form (shape) to the corresponding type on wet films, show scarcely any indications of such a structural differentiation of the cytoplasm. Only in one or two individuals, which are relatively rather wide, can traces of the bands be made out (cf. fig. 11). And none of these forms on wet films shows any signs of this peculiarity, the cytoplasm being practically homogeneous; this fact is, we think, most instructive. We have come to the conclusion that this appearance is, to a large extent, artificial, and chiefly the result of a certain flattening-out on the "dry" Giemsa-stained smears. The narrower and more compact the general cytoplasm of the parasite, the less conspicuous is this band-like arrangement.

It may be asked, Why was this condition found very frequently in the large forms of T. fringillinarum, which were figured as normal? It must be recalled in explanation that the preparations on which the account of these forms in the latter trypanosome was based differed in two respects from those we have of the parasites from the little owl. In the first place, no wet films of T. fringillinarum were obtained. Secondly, the smears were made from the peripheral blood, and were, of course, evenly spread out and thin. On the other hand, in the case of the trypanosome with which we are now concerned, all our preparations showing these large individuals are made from the bone-marrow, and the smears are not nearly so thinly spread out. In fact, the best fixed examples of these large forms are those which occur in the neighbourhood of clumps and masses of cells.

It is fairly certain, therefore, that in the blood-smears containing T. fringillinarum, these large blue parasites were, while really not distorted, nevertheless sufficiently flattened-out to produce this effect of bands in the cytoplasm. At the time, however, there was no reason why one should have con-

cluded this to be the case. If the figures given in the preceding memoir of the large forms of T. fringillinarum are compared with figs. 10 and 24 accompanying of the corresponding type of the parasite of the little owl, it will be noted that the general cytoplasm in the former appears distinctly wider in proportion to the length of the body than in the latter trypanosome, although this is really a larger (longer) species. It may be mentioned that in one of the figures (fig. 48, pl. 5) which Zupitza (loc. cit.) gives of the large trypanosome identified by him with "T. ziemanni" from Eurystomus afer, showing an individual that is manifestly flattened out, the cytoplasm also shows distinct bands.

As regards the significance of this appearance we think it is probable that the clearer, lighter longitudinal zones, which are usually the narrower, may correspond to the position of the myonemes, though they do not, of course, actually represent them. The myonemes themselves are most probably fine but definite lines; apparently they are not easily demonstrable in Giemsa-stained preparations. One of us (13) was fortunate to secure a preparation of T. percæ, made by the wet method, which showed the myonemes well, but even in the case of the large T. raiæ, in iron-hæmatoxylin-stained films, we were unsuccessful (14) in seeing them; also in none of our wet preparations of the larger forms of the trypansome of the owl have we been able to make them out.

Of the three types or phases of the trypanosome above described, which occur together in the bone-marrow, and there alone, the small forms are the most numerous, the big blue individuals are distinctly less frequent, while the slender, medium-sized type, in its most fully developed condition with the long, narrow aflagellar prolongation is least common, and occurs somewhat scantily.

As already indicated, these three phases can be definitely connected with one another by means of transitional forms which occur. Thus the medium-sized, slender parasites arise from the small ones by growth and extension of the

body, principally in the direction of length ; at the same time the aflagellar prolongation becomes conspicuously developed.

A regular series of intermediate stages is seen, for example, in figs. 4, 18, 17, 6 and 19. The dimensions of these individuals are as follows: Fig. 4, (*a*) total length 35 μ, (*b*) width $2\frac{1}{2}\,\mu$, (*c*) free flagellum 11 μ, (*d*) aflagellar part 4 μ; fig. 18, (*a*) 40 μ, (*b*) $2\frac{3}{4}\,\mu$, (*c*) 16 μ, (*d*) 3 μ; fig. 6, (*a*) 39 μ, (*b*) 3 μ, (*c*) $9\frac{1}{2}\,\mu$, (*d*) 5 μ; fig. 17, (*a*) 42 μ, (*b*) $3\frac{1}{2}\,\mu$, (*c*) 12 μ, (*d*) 4 μ; fig. 19, (*a*) 43 μ, (*b*) 4 μ, (*c*) 11 μ, (*d*) $5\frac{1}{2}\,\mu$. It will be noticed that the length of the free flagellum may vary not inconsiderably and apparently indiscriminately in these forms. This point was referred to in describing the cultural forms of T. fringillinarum ; and we think it is most probable that the explanation given in that case holds here also, namely, that the different length of the flagellum in what are otherwise similar individuals is chiefly the result of the unequal splitting of this organella in dividing parasites.

In T. fringillinarum, it may be remembered, certain of the small forms occurring in the bone-marrow, which were broader and more stumpy than the others, were found showing unmistakably commencing division (cf. fig. 54 of the earlier memoir). Hence, as regards the corresponding small forms from the little owl, it is most likely that they also divide by binary fission, although apparently most infrequently. We have not been able to secure as marked indications of the process as were obtained in T. fringillinarum, but we have found individuals which are broader or stouter than the rest, some of which showed the kinetonucleus double (fig. 16). Just as in the case of the chaffinch-parasite, these small trypanosomes are the only type of form in which we have been able to find even a hint of binary division.

Next, with regard to the large "blue" parasites, these also can be undoubtedly linked up to medium-sized, slender forms by intermediate stages, such as those shown in figs. 9 and 23. The measurements of these two individuals are as follows: Fig. 9, length 43 μ, width $3\frac{1}{4}\,\mu$, free flagellum 7 μ, aflagellar prolongation 8 μ; and fig. 23, length 39 μ (at

least),[1] breadth $4\frac{1}{2}$ μ, free flagellum 7 μ (at least), and aflagellar portion 6 μ. It will be seen that from medium-sized forms, such as those of figs. 6, 17 and 5 (which have not attained the extremely elongated appearance presented by this type, when fully developed), it is a very slight step to the "blue" forms of figs. 9 and 23.

The latter type arises, we should say, principally by an increase in bulk or density of the general cytoplasm of the body, which results in a distinct alteration in its staining reaction (chiefly by Giemsa). It is important to note that this in no way involves a contraction in length, i.e. there is no shortening of the body to compensate for increased stoutness. On the contrary, in the larger individuals the whole body, including the aflagellar portion, is found to have increased in length as well as in width or bulk. We shall have to refer again to this point when subsequently criticising Zupitza's paper.

In the later owls (Nos. 19 and 23) the trypanosomes are in a quite different phase—that is to say, the parasites are in what we regard as the summer condition. We have not observed any individuals either of the slender, medium-sized variety, or of the large blue type; both these forms appear to be entirely absent. Neither do most of the parasites present in these later owls quite correspond to the small forms occurring in the earlier birds, though in all probability they are developed from the latter type.

The summer forms of trypanosome (figs. 12, 13, 29–31, 40–42) are all fusiform and stumpy in character, with the kinetonucleus situated near the abruptly pointed aflagellar end; nearly all the individuals observed, however, are distinctly larger than the small forms above described. Moreover, the body, instead of being a fairly slender spindle, is, in most cases, comparatively thick or stout in proportion to its length. This character may appear to be very marked, both

[1] The total length of the parasite, as also the length of the free flagellum, is probably a few μ longer, since the end of the flagellum pasess over the nucleus of a blood-cell, where it cannot be traced.

among the smaller and the larger individuals met with, so much so that many of these parasites show in stained preparations what has been described as a leaf-like appearance or shape (figs. 12, 30, and 31). This is seen chiefly in smears made from the peripheral blood, and is only exceptionally found in the case of individuals on smears made from the bone-marrow. Here also we have come to the conclusion that this wide, leaf-like appearance is largely or almost entirely due to the (artificial) flattening-out of thick, fusiform parasites on thinly spread smears. This view is borne out by a comparison of this type of parasite as it occurs in films prepared by the wet method and stained by iron-hæmatoxylin. All the parasites observed have the form of a stout spindle (figs. 40–42); but no leaf-like individuals have been seen on wet films. Moreover, in the leaf-like forms the trophonucleus appears more or less transversely elongated (figs. 12, 29, 30), which is never the case in any other form of the parasite; compare Minchin (**13**, p. 17) on similar forms of Trypanosoma percæ. Hence, in arriving at a correct estimate of the proportions of the summer type of the parasite, such flattened-out individuals are best left out of consideration, since their breadth probably appears considerably greater than is actually the case.

Fig. 13 shows the typical appearance of a trypanosome of this stout, fusiform type, this being a fairly large individual. Its length is 32 μ, greatest breadth or thickness (including the undulating membrane) $4\frac{3}{4}$ μ, and the length of the free flagellum 9 μ. Rather smaller parasites are seen in figs. 25 and 26, the former being 30 μ long, $4\frac{1}{4}$ μ broad, and the free flagellum 7 μ, while the latter is 34 μ long, accounted for by the much longer flagellum of 12 μ, and $4\frac{1}{2}$ μ broad. Of the flattened, more leaf-like individuals those of figs. 31 and 30 correspond in reality closely, we have no doubt, to the above-mentioned parasites. The former is 32 μ long, the free flagellum being $6\frac{1}{2}$ μ, and its width appears to be $6\frac{1}{2}$ μ; the latter is $29\frac{1}{2}$ μ long, the flagellum being 8 μ, while the width is apparently as much as $7\frac{1}{2}$ μ. This latter parasite

shows, it will be noticed, the curious chain of granules running parallel to the flagellar border, which was frequently observed in the case of the corresponding forms of T. fringillinarum. The smallest trypanosome of this type which has been observed (fig. 29) also happens unfortunately to be on a thin blood-smear,[1] and is leaf-like. It is only $23\frac{1}{2}\,\mu$ long, the free flagellum being $6\frac{1}{2}\,\mu$, and it appears to be as much as $6\frac{1}{2}\,\mu$ wide! Individuals as small as this are very exceptional; the majority appear to be of much the same size, having an average length of about $30\,\mu$, and not differing greatly from the dimensions given above.

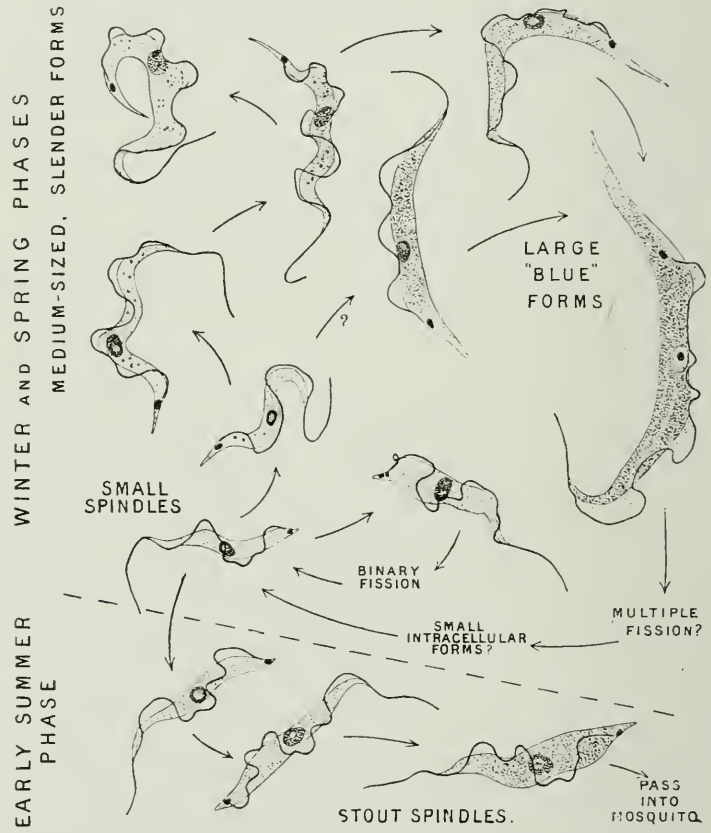
Comparing now this type of form as it occurs on wet films, we find, as already indicated, that all the parasites are fairly uniform in size and appearance. They show well the typical thick, fusiform shape of the body. They also exemplify another important point, namely, the considerable shrinkage in size which is undergone by the trypanosomes in the course of preparing the wet films. The total length averages about $23\,\mu$, the individual of fig. 42, for instance, being $25\,\mu$ long by $4\frac{1}{4}\,\mu$ broad, while that of fig. 41 is $20\frac{1}{2}\,\mu$ by $3\frac{3}{4}\,\mu$. We have no hesitation in comparing these forms with the average-sized ones of the dry series—say, for instance, figs. 13 and 30 respectively. It might be thought, perhaps that the parasites of figs. 40–42 corresponded rather to the very small individual of fig. 29. We certainly do not think so. We have only found such a small form in that one case, the average size being considerably larger (longer), and the wet films were made at the same time as the Romanowsky ones. Further, as was shown above, there is relatively quite as great shrinkage in what are undoubtedly medium-sized slender forms and large "blue" ones (i. e. types found in the earlier birds) on wet films.

A feature in which the stout spindles differ markedly from the slender small forms is the much larger size of the tropho-

[1] On one or two thick blood-smears which were made, nearly all the parasites have the more typical form, only a few individuals in the thinner parts tending to be flattened out.

nucleus, particularly of the karyosome. This is well brought out by iron-hæmatoxylin-stained films (figs. 40–42), but it is also seen in those Giemsa-stained individuals in which the

Text-fig. 1.



Scheme showing the different types of form of Trypanosoma noctual and their connection with one another.

karyosome appears as a clear area in the nucleus (figs. 12 and 13).

General Considerations.

The principal conclusion at which we have arrived in this paper is that all the different forms of trypanosome in the

blood of Athene noctua belong to one and the same species, for which we use the name Trypanosoma noctuæ, Schaudinn, in spite of the differences between Schaudinn's figures and ours. Above is a scheme showing the connection between the different types of individual here described.

The stout spindles, which are the only forms we have found in the summer condition of the trypanosome, and which occur, it will be remembered, also in the general circulation, are undoubtedly a transmissive phase of the parasite; on passing into a mosquito (Culex) they give rise, as will be shown in a subsequent memoir, to the developmental forms characteristic of the Insectan host. It is interesting and important to note that an exactly similar state of affairs was found to be present in the trypanosome of the chaffinch in its summer phase (cf. Case B in the former memoir).

As indicated in the scheme, these stout spindles arise, we feel practically certain, from individuals of the small fusiform type, such as occurred in the earlier birds, their development being along rather a different line from that leading to the medium-sized and large "blue" types (the early spring phases). Individuals such as that shown in fig. 27 are clearly transitional between the small type and the fully-developed stout spindle. While the parasite increases in length to a certain, but not very great extent, the principal direction in which growth or development takes place is a pronounced increase in thickness or stoutness of the body (cf. figs. 32 and 33 and 40 and 42 respectively, from wet films).

Zupitza (loc. cit.) has, it seems to us, a quite erroneous idea about the origin of these stout forms (and, what are the same thing, the leaf-like forms). He considers them as being later (older) stages of long, slender (so-called "spirochætiform") parasites. They are regarded as being developed by a process of thickening or swelling of the body-protoplasm, chiefly in the middle; concurrently the kinetonucleus is pushed further towards the aflagellar end, and the delicate

aflagellar prolongation is gradually lost, this region of the parasite ultimately taking on the short, abrupt appearance characteristic of the stout spindles. We certainly have never found any indication of such a process, either in the trypanosome of the little owl or in T. fringillinarum from the chaffinch. In those types of form which develop a marked aflagellar prolongation, this becomes distinctly more prominent with the growth of the individual, corresponding with the increase in size of the rest of the body. This is the case both with the medium-sized, tapering forms (which were termed "definitive" forms in the first memoir [18]), and the massive "blue" forms, as is clearly shown by a comparison of the series of figures, both of the parasite of the owl and that of the chaffinch. On the other hand, in the case of both the distinct trypanosomes with which these studies have been concerned, the stout spindles can be readily connected by transitional forms with the small fusiform type (cf. particularly the figs. 5, 6, 42–44 of T. fringillinarum, which show a complete series of intermediate stages). Another point to be noted is that the larger individuals of the slender, tapering type have considerably more bulk than the smaller individuals of the stout spindle type, and could by no possibility become the latter. Zupitza's account gives, indeed, a somewhat confused idea of the different phases of Avian trypanosomes and their relation to one another. This is partly due to the fact that he compares trypanosomes from quite different hosts as if they were all phases in the life-cycle of one and the same parasite, without having regard to possible differences in size of the various forms, instead of studying the same form in the same host through different seasons of the year.

We have not been able to observe the condition or phase in which the trypanosomes occur in the owl in the early autumn, not having had any infected birds at this season. Hence, we do not know what becomes of the stout spindles, which do not pass into the Insectan host. As was discussed in the case of T. fringillinarum, we think it quite likely such forms

may develop further into large "blue" forms; these were found in the general circulation in the autumn in the case of T. fringillinarum.

The chief, and, indeed, about the only point of difference between the trypanosome of the little owl and T. fringillinarum, so far as we have observed the two parasites, is that in the latter species the massive forms were not found in association with the small forms and the medium-sized ("definitive") individuals in the early spring, as in the former parasite. It is quite possible, however, that these forms could be found at that period in T. fringillinarum also.[1] Hence we do not now consider that there is much to be gained by continuing to distinguish the medium-sized slender individuals as "definitive" forms. In the case of the owl-trypanosome this phase does not appear to be so prominent or persistent as was found to be the case in T. fringillinarum. It was mentioned in describing that species that the "ordinary" forms, as they were regarded, might pass later into the massive "blue" type. As we have shown above in the present case there is undoubtedly a transition from medium-sized slender forms to the large blue type; though we are uncertain whether the former phase in its most fully developed condition (cf. figs. 7 and 8) undergoes this further development.

From the marked correspondence as regards the different types of form which we have found in the case of two species from widely different hosts we venture to think that the scheme drawn up on p. 176 may be regarded as applicable in the main to other Avian trypanosomes, and may perhaps be taken as indicating typical phases in the life-cycle occurring in the bird which are common to most species. Reading Zupitza's paper in this light we find that several points of agreement with our results are shown by the parasites with which he worked. The same types of individual are described

[1] Since this paper was sent to press, one of us (H. M. W.) has found these large forms in a chaffinch infected with I. fringillinarum early in May.

or figured under one name or another, and from one or more birds. The true stout spindles, it may be noted, which type is distinguished by Zupitza as "T. avium minus" occurred in most instances alone, just as we found to be the case.[1] Massive blue forms were also met with (termed in one case "T. avium majus" and in another T. ziemanni).

From the descriptions which have been given of the trypanosome of the little owl and of T. fringillinarum in this and the preceding memoir, it will be clearly seen, we think, that these various names, such as T. avium minus, T. avium majus, etc., can be regarded only as general designations for different types or phases which occur in the life-cycle of, at any rate, many species of Avian trypanosome. They do not represent distinct and independent forms or varieties. A further very important point brought out is that this applies also to the type which has been hitherto distinguished as T. ziemanni. "T. ziemanni" is really only the large "blue" phase of T. noctuæ, the trypanosome parasitic in Athene noctua. This is equally true, we have no doubt, for the species parasitic in Syrnium aluco, whether that is to be considered also as T. noctuæ, or as being a distinct species.[2] Mayer (**12**), in his recent paper on the parasites of this latter owl, which will be more fully dealt with in a subsequent memoir, figures trypanosomes which belong both to the stout spindle type and to the large massive forms, the latter being regarded as "Leucocytozoon-forms," i. e. as equivalent to Schaudinn's T. ziemanni.

As we stated in the earlier part of this paper we have seen nothing in the case of the parasites of the little owl to lend any support to the view that these large trypanosomes are actually connected with the Leucocytozoon ziemanni. In the first place the latter parasite, in its large form, always

[1] Unfortunately we cannot gather from Zupitza's account whether his birds were all examined at the same season of the year, or at different periods.

[2] If it is a distinct species. to it belongs the name T. avium, as emended by Laveran (**6**).

occurs in the gametocyte phase, i. e. in the character of male or female individuals which give rise only to the sexual elements. Moreover, we have never found any corresponding sexual difference among the massive trypanosomes which might indicate that they were of male or female character. They never show the marked differences in staining reaction presented by the Leucocytozoon.

We may repeat, therefore, that "T. ziemanni" is to be regarded only as a phase or type of form of T. noctuæ, just as similar large forms occur in T. fringillinarum; and again, for instance, in the trypanosome parasitic in Eurystomus afer (allied to the roller-bird), where they are also regarded by Zupitza (loc. cit.) as "Leucocytozoon-forms" and designated "T. ziemanni"![1]

In view of the general agreement which we have found between the different types of T. noctuæ and those of T. fringillinarum, why, it may be asked, do we not regard both these parasites, for instance, as belonging to the same species? We feel practically certain that they are quite distinct forms for the following reasons: First, on the ground of their occurrence in very different birds, the hosts being respectively little owls (and perhaps also other owls), and chaffinches (and also redpolls and perhaps other Fringillinæ). Secondly, we have on several occasions inoculated a little owl, free from any blood-parasites, with cultural forms of T. fringillinarum, but have been unsuccessful in obtaining any development of the trypanosomes in the bird. Lastly, on morphological grounds, the various types of form of T. noctuæ appear to attain a slightly but distinctly larger size than do the corresponding types of T. fringillinarum so far as we can judge from our own observations. This is seen from the following table comparing the two sets of forms:

[1] In any case, it is most unlikely that this parasite, occurring in a quite different bird, would be the same species as Schaudinn's "T. zeimanni."

Type of form.	T. fringillinarum.	T. noctuæ.
A. Small forms (slender spindles), smallest individuals observed	Total length, 25 μ. Greatest breadth, 3¼ μ	Total length, 26½ μ. Greatest breadth, 3½ μ.
B. Medium-sized, slender forms (so-called "definitive" forms), average size of fully developed individuals	Total length, 41–45 μ Greatest width, 4½–5 μ	Total length, 44–47½ μ. Greatest width, 5–5½ μ.
C. Large, massive "blue" forms, average size of largest individuals	Total length, 45–48 μ Greatest breadth, 5¾–6½ μ (probably slightly flattened out—see text)	Total length, 54–60 μ. Greatest breadth, 5¼–6 μ.

The Lister Institute,
April, 1911.

References to Literature.

1. Aragao, H. de B.—"Ueber den Entwickelungsgang und die Uebertragung von Hæmoproteus columbæ," 'Arch. Protistenk., xii, 1908, p. 154, pls. 11–13.
2. Chagas, C.—"Ueber eine neue Trypanosomiasis des Menschen; Studien über Morphologie und Entwickelungscyclus des Schizotrypanum cruzi n.g., n.sp.," 'Mem. Inst. Oswaldo Cruz,' i, 1909, p. 1, pls. 9–13.
3. Dutton, J. E., Todd, J. L., and Tobey, E. N.—"Concerning Certain Parasitic Protozoa observed in Africa," 'Mem. Lvpl. Sch. Trop. Med.,' xx, 'Ann. Trop. Med. Parasitol.,' i, 1907, p. 287, pls. 20–32.
4. Fantham, H. B.—"Observations on the Parasitic Protozoa of the Red Grouse," 'Proc. Zool. Soc.,' ii, 1910, p. 692, pls. 59–61.
5. Hartmann, M.—"Notiz über eine weitere Art der Schizogonie bei Schizotrypanum cruzi," 'Arch. Protistenk.,' xx, 1910, p. 361.
6. Laveran, A.—"Sur un Trypanosome d'une chouette," 'C. R. Soc. Biol.,' lv, 1903, p. 528.
7. ——— "Contribution a l'étude de 'Hæmamœba,' ziemanni," t. c., p. 620.
8. Mathis, C., and Léger, M.—"Leucocytozoon de la poule," op. cit., lxvii, 1909, p. 470.

9. ——— ——— "Recherches sur le Leucocytozoon de la poule," 'Periodicité des formes sexuées dans le sang," t. c., p. 688.

10. ——— ——— "Sur un nouveau Leucocytozoon de la poule," op. cit., lviii, 1910, p. 22.

11. ——— ——— "Nouvelles recherches sur Leucocytozoon sabrazesi et L. caulleryi de la poule domestique du Tonkin," 'Bull. Soc. Path. Exot.,' iii, 1910, p. 504.

12. Mayer, M.—"Ueber ein Halteridium und Leucocytozoon des Waldkauzes und deren Weiterentwickelung in Stechmücken." 'Arch. Protistenk.,' xxi, 1911, p. 232, pls. 22 and 23.

13. Minchin, E. A.—"Observations on the Flagellates Parasitic in the Blood of Fresh-water Fishes." 'Proc. Zool. Soc.,' i, 1909, p. 2, pls. 1–5.

14. ——— and Woodcock, H. M.—"Observations on Certain Blood-parasites of Fishes occurring at Rovigno." 'Quart. Journ. Micr. Sci.,' 55, 1910, p. 113, pls. 8–10.

15. Rosenbusch, F.—"Trypanosomen-Studien," 'Arch. Protistenk.,' xv, 1909, p. 263, pls. 25–27.

16. Schaudinn, F.—"Generations- und Wirthswechsel bei Trypanosoma und Spirochæta," 'Arb. kais. Gesundhtsa.,' xx, 1904, p. 387.

17. Wenyon, C. M.—"Report of Travelling Pathologist and Protozoologist." 'Rep. Wellcome Research Lab.,' iii, 1908, p. 121, pls. 9–16.

18. Woodcock, H. M.—"Studies on Avian Hæmoprotozoa: (1) On Certain Parasites of the Chaffinch (Fringilla cœlebs) and the Redpoll (Linota rufescens)," 'Quart. Journ. Micr. Sci.,' 55, 1910, p. 641, pls. 27–31.

19. Zupitza, M.—"Beitrag zur Kenntniss der Vogel- und Fisch-trypanosomen Kamerund," 'Beihefte Arch. Schiffs- u. Tropen-Hyg.,' xiii, 1909, p. 100, 6 pls.

EXPLANATION OF PLATES 20 AND 21,

Illustrating Observations by Messrs. E. A Minchin, M.A., F.R.S., and H. M. Woodcock, D.Sc., on "The Trypanosome of the Little Owl (Athene noctua), with Remarks on the other Protozoan Blood-parasites Occurring in this Bird."

[All the figures relate to Trypanosoma noctuæ, and are magnified 2000 times linear. We are indebted to Miss Rhodes for kindly drawing and colouring most of the figures on Pl. 20, and for drawing two or three of those on Pl. 21.]

PLATE 20.

Figs. 1–11 are from owl 8; figs. 12 and 13 from owl 23.

(All the figures are from preparations stained by Giemsa.)

Figs. 1–3.—Small forms (small spindles).

Fig. 4.—Transitional form from small fusiform type to slender, medium-sized type.

Figs. 5 and 6.—Young individuals of the slender, medium-sized type (not fully developed).

Figs. 7 and 8.—Fully developed individuals of the medium-sized form; the individual of fig. 7 is probably a little flattened.

Fig. 9.—Small individual of the massive "blue" type (c f. with figs. 5 and 6).

Figs. 10 and 11.—Large individuals of the massive "blue" type; the latter shows indications of the longitudinal bands in the cytoplasm.

Figs. 12 and 13.—Stout spindles (early summer phase); the former is a typical, full-sized individual; the latter is flattened out (so-called leaf form).

PLATE 21.

Figs. 14–17, 19 and 20, 22–24, 32–36, 38, 39 and 44 are from owl 8; figs. 18, 21, 37 and 43 from owl 13; figs. 25 and 26 from owl 19; and figs. 27–31, 40–42 from owl 23.

(Figs. 14–31 are from preparations stained by Giemsa.)

Figs. 14 and 15.—Small spindles.

Fig. 16.—Small spindle, just commencing division; there are two kinetonuclei, and apparently the karyosome in the trophonucleus has also divided.

Figs. 17–19.—Intermediate stages in the growth of the medium-sized slender type.

Figs. 20 and 21.—Fully developed medium-sized forms.

Figs. 22 and 24.—Massive "blue" forms.

Fig. 23.—Small individual of the same type.

Figs. 25, 26, and 28.—Stout spindles.

Fig. 27.—Transitional form from small fusiform type to stout spindle.

Figs. 29–31.—Stout spindles, more or less flattened-out (leaf-like); the individual of fig. 29 is the shortest of this kind found.

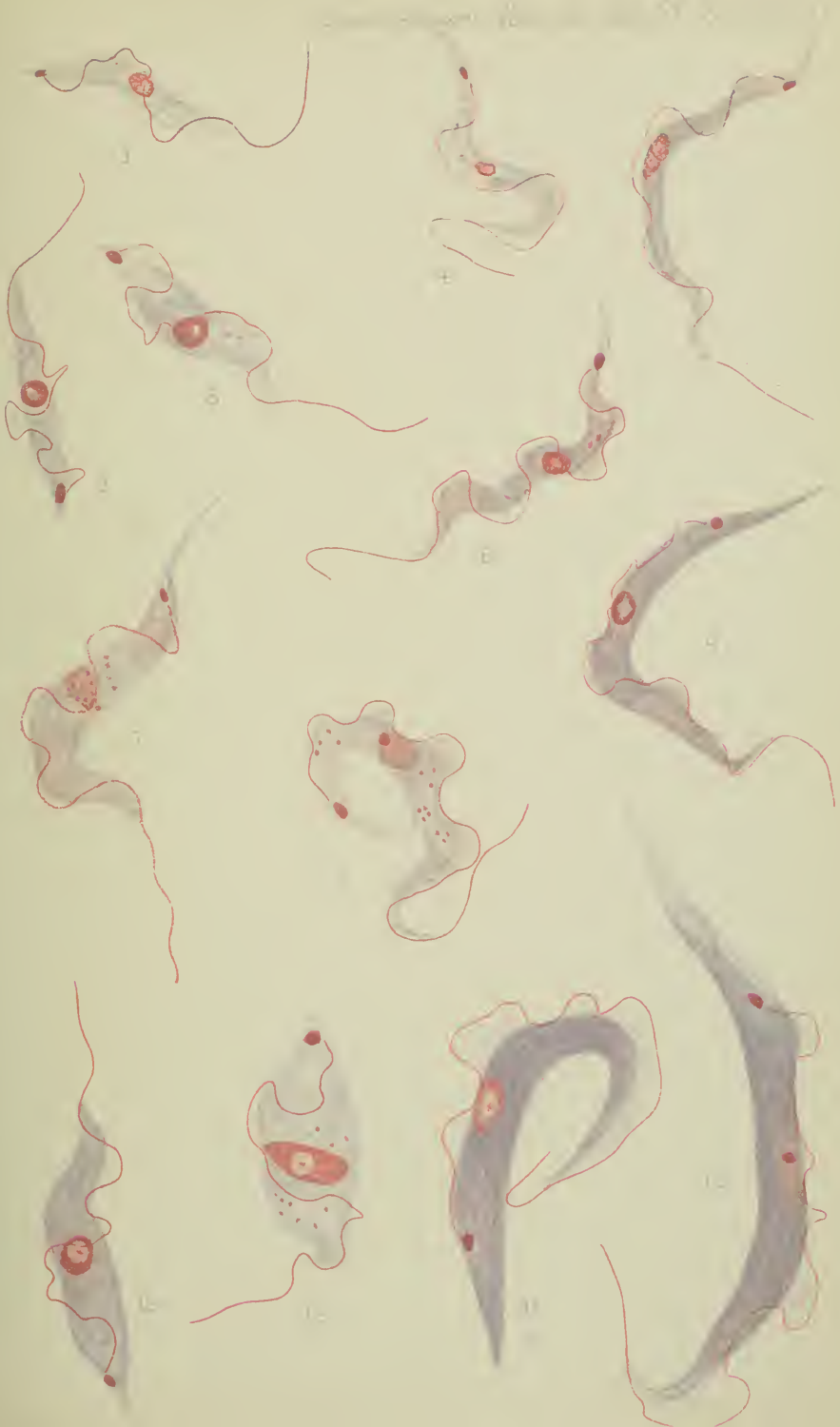
(Figs. 32–44 are from preparations stained by iron-hæmatoxylin.)

Figs. 32–35.—Small spindles.

Figs. 36–39.—Medium-sized slender forms.

Figs. 40–42.—Stout spindles.

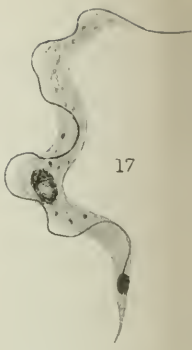
Figs. 43 and 44.—Massive "blue" forms.




14.


15.


16.


17


25.


27.


26.

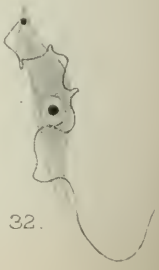

28.

24.


33.

29


30


32.

31


21.
20.
19.
23
40
44.
34.
39
41.
38.
37
42
43.
36

[*From the* Proceedings of the Zoological Society of London, 1911.]

[Published September 1911.]

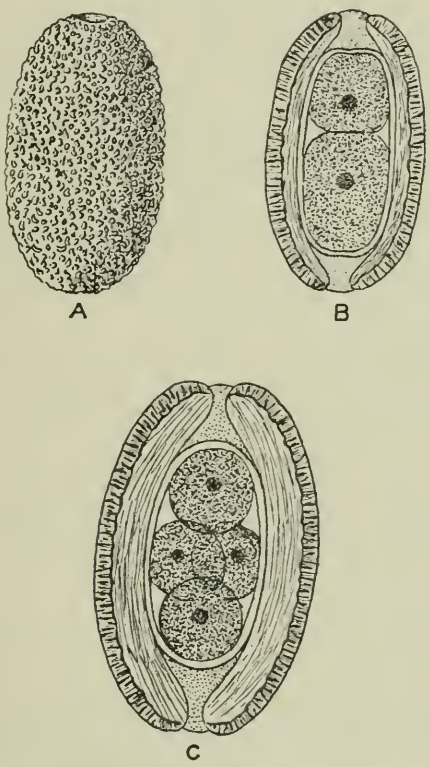
On a unique Pathological Condition in a Hare.

(Text-figure 165.)

Dr. William Nicoll, M.A., F.Z.S., exhibited some preparations from a Common Hare (*Lepus europæus*), which showed an interesting and unique pathological condition. The hare was obtained by W. Raphael Muckley, Esq., and sent by him to the British Museum, whence it was forwarded to the Lister Institute of Preventive Medicine. The manner in which the hare died was somewhat remarkable. It was observed by Mr. Muckley to pitch violently out of a hedge on to the roadside where it lay struggling, and it died about three hours later. To him the symptoms seemed to point to poisoning. At the *post mortem* examination the liver was found to be extensively invaded with small whitish chalky deposits of various sizes, especially conspicuous on the posterior surface. There was also a considerable amount of chronic inflammation around the liver, with adhesions to the diaphragm. On section the liver was friable and gritty. The concretions were amorphous and insoluble in acid. The bile-ducts showed some fibrous thickening. On microscopic examination of a scraping from the liver, numerous ova of peculiar structure were seen. They were fairly uniform in size, measuring ·057 × ·033 mm. At first sight they bore a considerable resemblance to the eggs of *Trichuris* (*Trichocephalus*) or *Trichosoma*, but on more careful inspection they presented one or two unusual features. The colour was much lighter, being greyish instead of brown. The shell consisted of two layers, the outer of which was marked with pronounced radial striations; the inner was

constituted of fine concentrically-arranged lamellæ. The two layers were not in close apposition but were separated by a narrow, somewhat irregular space. The egg is thus provided with a double shell. At each pole of the egg there was a small circular aperture, piercing both layers of the shell. The external surface of the shell presented a well-marked papillated appearance, the papillæ being irregularly rounded. Inside the shell

Text-fig. 165.



Eggs of a Nematode worm from the liver of a Hare.

A. External surface of egg. B. Optical section of egg: two-celled stage.
C. Egg in four-celled stage with thickened inner wall: from vagina.

there was a thin, continuous membrane investing the egg. The polar apertures were each closed by a plug of homogeneous material which was separate and distinct from the membrane investing the egg-cells. They completely filled the apertures but did not bulge beyond them. The eggs were mostly in the two-celled stage, but a number of them showed four cells.

[3]

From the appearance of the eggs it is obvious that the lesions were due to a Nematode worm of the *Trichosoma* group. Search was accordingly made and a number of worms were obtained. In every case, however, they were fragments lacking the anterior and posterior extremities. The longest specimen measured 27 mm. It was a female, full of eggs, and it was very slender. It was narrowest at the anterior end (·14 mm.), the breadth increasing gradually towards the posterior end where it was ·23 mm. The cuticle was marked throughout with fine annular striations. The vagina was of considerable length but no genital aperture was seen, so that it must have been far forward. In the vagina a number of eggs were present, which were much larger than those first seen. They measured ·070–·078 × ·040–·045 mm. The shell, too, was much thicker, the increase being almost entirely due to a great thickening of the inner layer. The polar apertures were somewhat reduced in size and the eggs were for the most part in the four-celled stage.

The lesions in the liver showed much resemblance to those seen in advanced stages of coccidiosis, a very common condition in rabbits. For this reason they might readily be diagnosed as such on cursory examination. This, so far as can be gathered, is the first record of such an infection of the liver of hares or rabbits by parasitic nematodes of this kind.

An analogous condition has not infrequently been met with in rats, and has been reported from Europe, India, and Australia. No record has been made of its occurrence in this country.

Whether this parasite in the hare is the same as that in the rat must remain doubtful, for the descriptions of the latter have hitherto not paid much attention to the character of the eggs beyond mentioning that they resembled those of *Trichosoma*.

An attempt is being made to hatch the eggs and to produce infection in rabbits and rats, but the development is extremely slow even at a constant temperature of 26° C.

In addition to the lesions in the liver the hare had a very heavy infection of *Trichostrongylus retortæformis* in the intestine and a slight intestinal infection with *Coccidium cuniculi*. The intestine and the appendix, moreover, showed a large number of small calcareous patches, but on examination nothing of a parasitic nature could be detected in the patches. This was of interest from the fact that I had already seen similar patches in the cæcum of a Variable Hare, sent me from the Society's Gardens. The liver, unfortunately, was not submitted for examination. That these patches might have something to do with the liver condition was not impossible, for they might be considered as degeneration following injury such as the passage of a worm through the intestinal wall.

The hare, further, showed signs of recent parturition and there was a septic condition of the uterus, and it must remain an open question as to whether death was due to this or to the liver disease.

[*From the* Proceedings of the Zoological Society of London, 1911.]
[Published September 1911.]

On Three New Trematodes from Reptiles.

By William Nicoll, M.A., D.Sc., M.B., F.Z.S.*

(Plates XXVII. & XXVIII.†)

The following notes were made on a collection handed over to me by the Prosector of the Zoological Society. In no case was the habitat noted, but from their affinities it may be presumed that the specimens all came from the lungs, the mouth, or the œsophagus. The specimens are interesting as forming an important addition to our knowledge of the large variety of forms which inhabit the air-passages and anterior end of the alimentary canal of reptiles and batrachians. To the taxonomy of this particular group Odhner has recently (1910) made a valuable contribution, which will be further referred to later.

The first species is from the Hog-nosed Snake (*Heterodon platyrhinus*), and I include it provisionally in the genus *Lechriorchis* Stafford, 1904. Like most of Stafford's genera, this genus is insufficiently defined, although the fact that he includes in it the well-described species *L.* (*Renifer*) *elongatus* Pratt, 1903, is something to go upon. It is, therefore, necessary to amend Stafford's definition somewhat, as follows.

Genus Lechriorchis Stafford, 1904.

Resembling *Renifer* Pratt, 1903, except in the following particulars. The intestinal diverticula extend a short distance *beyond* the testes; the genital aperture is further from the edge of the body, midway between it and the pharynx; there is a well-developed vagina.

These constitute, as far as appears, the only differences between the two genera.

Lechriorchis validus, sp. n. (Plate XXVII. figs. 1–5.)

This is a species of moderate size, measuring 3·6–7·4 mm. in length by ·9–1·4 mm. in breadth. The smallest specimens had just begun to produce ova, so that the minimum adult size is probably about 3 mm. The body is elongated, the length being 4–5 times the breadth. The latter is comparatively uniform, but the tail is distinctly pointed. The anterior end is more rounded, while there is a tendency for a slight narrowing to occur about or behind the ventral sucker. In young specimens the body is flattened, but it becomes much thicker as the uterus increases in size. In transverse section an adult specimen presents a strongly convex dorsal surface and a flat or slightly convex ventral surface.

* From the Lister Institute of Preventive Medicine, London.
† For explanation of the Plates see p. 686.

The cuticle is fairly thick, somewhat deciduous, and is studded throughout its whole extent by stout salient spines. These are regularly arranged, and become sparse towards the posterior end. The underlying musculature has the usual formation.

In an average adult specimen of 6·5 mm. length the oral sucker has a diameter of ·45 mm. It is subterminal, globular, and its aperture has a marked muscular rim. The thickness of its wall is about ·13 mm. In the same specimen the ventral sucker measures ·66 mm. It is also globular, slightly flattened, and its wall is ·18 mm. thick. The sucker ratio is, therefore, very approximately 2 : 3. The diameter of the sucker relative to the body-length diminishes as the animal increases in size. Thus, in the smaller specimens the ventral sucker is $\frac{1}{7}$ of the body-length, while in the largest it is only $\frac{1}{11}$. The ventral sucker is situated about $\frac{1}{3}$ of the body-length from the anterior end. In this respect, again, the young specimens differ from the older ones in that they have the ventral sucker relatively further back.

There is a very short prepharynx; usually the pharynx is contiguous with the oral sucker, and it measures ·23 × ·17 mm. The œsophagus is about $\frac{3}{4}$ of the length of the pharynx, although it is longer in young specimens. The intestinal bifurcation takes place well in front of the ventral sucker. The intestinal diverticula run almost parallel to the edges of the body. They bend in a little just behind the ventral sucker, but they are pressed out again by the testes. At their termination, just behind the posterior testis, they usually turn in a little. Their ends are about $\frac{2}{5}$ of the body-length from the posterior end. They are comparatively narrow tubes, and their wall is crinkled on the inner side. The outer side is plain. They are lined by a layer of low epithelium. The œsophagus has the usual cuticular lining. Throughout their whole extent the diverticula are somewhat ventral in position.

The excretory system is characteristic and agrees with Odhner's supposition as to its form in the group to which this species belongs. The vesicle consists of a fairly broad main stem, opening at the tip of the tail and passing forwards to the shell-gland, where it divides into two limbs. These diverge to form a Y, and each passes over the edge of the ventral sucker and terminates halfway between the sucker and the intestinal bifurcation. The main stem is pressed close to the dorsal surface, but the limbs are not quite so dorsal in position. They eventually come to lie close up to the intestinal diverticula and separate these from the uterus and cirrus-pouch respectively (Pl. XXVII. fig. 4). The main stem is much compressed dorso-ventrally, but the limbs are round or compressed transversely. In addition to this central system, however, numerous lateral twigs are given off from the stem and limbs. These all radiate outwards towards the edges of the body, dividing and subdividing and eventually forming an intricate anastomosis (Pl. XXVII. fig. 3). The entire lateral fields of the body are thus filled with a complex network of excretory tubes, and the amount

of parenchymatous tissue is very much reduced. This is more particularly the case in the post-acetabular region. It gives rise to a reticulated appearance, which is strikingly seen in young specimens (Pl. XXVII. fig. 1). In older specimens it is obscured by the growth of the uterus.

The genital glands are difficult to distinguish in the adult, but are easily seen in younger specimens. The testes lie not very far behind the ventral sucker, near and internal to the ends of the intestinal diverticula. They are obliquely situated, the left testis being half its diameter in advance of the right, and they are separated from each other by the uterus. In a young specimen, the uterus being narrow, they lie quite close together, but they are pressed further and further apart by the growth of the uterus. They are flat, elongated oval bodies measuring at least ·8 × ·4 mm. Their outer margin is plain, but their inner margin is indented in one or more places, so that the outline is somewhat irregular. At first they lie almost flat in the body, but the expansion of the uterus pushes their inner border towards the dorsal surface so that eventually they are considerably tilted.

The genital aperture is situated on the left side midway between the pharynx and the edge of the body. It is always on the level of the pharynx. The cirrus-pouch is of considerable length, and is a conspicuous object. It is elongated, somewhat slender, and extends to the middle of the ventral sucker. Its wall is remarkable for the great development of the longitudinal muscular fibres, which are stout and very prominent. The circular fibres are much smaller. Within the pouch there is a more or less highly convoluted vesicula seminalis. Usually it is simply bent double (Pl. XXVII. fig. 2), but frequently it is much more twisted. It is small compared with the size of the cirrus-pouch. It is not much dilated, and it is connected with the pars prostatica by a narrow duct. The pars prostatica is of relatively great length. It is an almost straight tube extending from a little in front of the ventral sucker to the point where the cirrus-pouch crosses the left intestinal diverticulum. It is uniform and fairly narrow. Surrounding it are numerous prostatic cells which fill up the greater part of the cirrus-pouch. The ductus ejaculatorius is short and narrow, and the exsertile, unarmed cirrus is not very long. The genital sinus is quite small.

The ovary is situated just behind the end of the cirrus-pouch, over the right posterior quadrant of the ventral sucker. Half of it lies beyond the sucker, and it frequently overlaps the adjacent intestinal diverticulum. It is an almost globular body, somewhat flattened dorso-ventrally, and is about half the size of the testes. Behind and internal to it lies a compact shell-gland, with a short ootype and a Laurer's canal, but no receptaculum seminis. Laurer's canal is short and opens dorsally in the middle line, about the level of the ovary. A small yolk reservoir lies dorsal to the shell-gland. The yolk-glands are of limited extent. They are entirely lateral and lie close to the outer side of the intestinal diverticula. On each side there are about half a dozen ill-defined

groups of follicles, which extend from midway between the intestinal bifurcation and the anterior edge of the ventral sucker to near the ends of the intestines. The initial part of the uterus is practically empty. In section it can be made out with difficulty as an extremely narrow tube, passing backwards from the shell-gland. Behind the right testis it widens out to form a receptaculum seminis uterinum, consisting of about four or five small dilatations. Further back a few ova appear, but the uterus still remains somewhat narrow. When it has nearly reached the posterior end of the body it turns abruptly on itself to form an ascending limb. Almost immediately this begins to dilate, and it has only proceeded a short distance before it almost completely fills the interior of the body. It passes forward, over and between the testes, crosses the ventral sucker, and terminates in a straight, thick-walled vagina, which is about half the length of the cirrus-pouch, and lies on the left side. The uterus thus consists of a descending and an ascending limb, the former being empty for the greater part of its length, and the latter being enormously dilated. The increase in size, therefore, takes place, not by an increase in convolutions, but by a great dilatation of the ascending limb. The ova are very numerous, rounded oblong in shape, and dark brown in colour. They have a large well-marked operculum. Many of them are more oval than oblong, and this gives rise to some variation in dimensions. From a large number of measurements the limits were found to be ·038–·045 mm. for the length, and ·018–·023 mm. for the breadth, and the average ·040 × ·021 mm. The extreme sizes observed were ·045 × ·018 mm. for the most oblong ova, and ·038 × ·023 mm. for the most oval.

No case of amphitypy was observed in any of the two dozen specimens forming the collection. The nearest approach was in one specimen where the testes were practically symmetrical, the left being a trifle behind the right, but the ovary and genital aperture were normal. In all the other specimens the position of these structures was exactly as I have described. The species, however, is extremely variable in one respect, namely, the posterior limit of the yolk-glands. Hardly two specimens agree in this respect. In some specimens they extend a short distance beyond the testes, in others they reach the middle of the posterior testis, and again in others they stop short of the testes. In addition, they are very frequently asymmetrical, extending further back on the right than on the left, or more rarely *vice versa*. The fact, however, that their anterior limit is constantly symmetrical induced me to consider a symmetrical posterior limit as the normal. In two specimens also, the intestinal diverticula were of unequal length, the left diverticulum being considerably shorter than the right, which was normal. The position of the genital aperture varied only very slightly, and most of the apparent variations were due to contraction. The length of the cirrus-pouch was practically constant, although in one specimen it extended nearly to the posterior border of the ventral sucker. The size of the ova was constant within the limits noted, and no increase in size takes

place as the animal grows older. The average size of the ova in young specimens was found to be the same as that in fully grown specimens, and I am inclined to view with some doubt Odhner's statement (2. p. 59) that the ova in *Renifer sauromates* Poir. increase in size as the animal grows older.

From *Lechriorchis elongatus* Pratt, this species is distinguished by having more unequal suckers, the ventral being decidedly larger, the yolk-glands being more extensive, being present some distance in front of the ventral sucker, and in having slightly larger eggs. From *L. primus* Staff., it appears to be distinguished by its much smaller eggs, its smaller ventral sucker, and probably in other respects.

The second lot of specimens consists of five from an Annulated Snake (*Leptodira annulata*). They bear a close superficial resemblance to *Lechriorchis validus*, but they do not belong to the same genus. The only genus to which they can at present be referred is *Ochetosoma* Braun, 1902, but they do not entirely agree with the definition of that genus as given by Braun. In internal anatomy they correspond quite closely, but they are not nearly so flattened as *O. monstruosum* Brn., the only species of the genus. The only other genus to which they could be referred is *Renifer* Pratt, 1903, but from that they differ radically in the configuration of the uterus.

Ochetosoma formosum, sp. n. (Plate XXVIII. figs. 6 & 7.)

The body is elongated and slightly flattened. The length is 3·7–5·7 mm., and the greatest breadth, about the ventral sucker, is 1·1–1·6 mm. The breadth is therefore rather less than $\frac{1}{3}$ of the length. The body tapers gradually towards each end.

In an average specimen (length 4·5 mm.) the oral sucker has a diameter of ·45 mm., *i. e.* $\frac{1}{10}$ of the body-length. It is globular and subterminal. The ventral sucker is situated 1·7 mm. from the anterior end. It is slightly oval, the transverse diameter being ·64 mm., and the longitudinal ·57 mm. The sucker ratio is therefore approximately 3 : 4.

The cuticle is extremely deciduous; in most of the specimens it was almost entirely stripped off. Only in one was it intact, and even then not completely so; from this specimen the presence of minute regular spines was determined.

There is a short prepharynx followed by an almost globular pharynx, measuring ·17 × ·16 mm. The œsophagus is about the same length as the pharynx (·2 mm.), and the intestinal bifurcation occurs well in front of the ventral sucker. The diverticula diverge pretty widely, and they terminate almost immediately behind the ventral sucker, the ends being somewhat turned in. They are narrow and irregularly dilated, but there is no crenation on their inner wall as in *Lechriorchis validus*. They are lined by low epithelium.

The excretory system is almost identical with that in *Lechriorchis validus*. The vesicle consists of a similar dorsal main stem, which

divides just behind the ovary into two limbs, which pass forwards a short distance in front of the ventral sucker. They are thus not so long as in the above mentioned species. The main stem, too, appears more expanded. There is the same system of secondary lateral branches which anastomose freely in the sides of the body.

The genital aperture is situated almost at the extreme left margin of the body, on the level of the posterior end of the pharynx. The genital sinus is very small. The cirrus-pouch is very like that of *Lechriorchis validus*, but it is shorter. It is usually disposed obliquely, and it terminates a short distance in front of the ventral sucker, from which its end is usually separated by a coil of the uterus. In one specimen it almost reached the sucker. It contains a small convoluted vesicula seminalis, ending in a narrow portion which runs into the pars prostatica. The latter is not so long as in the foregoing species, and it is more expanded, especially at its posterior end. The prostatic cells are numerous. There is a short ductus ejaculatorius, and an unarmed cirrus of moderate length. The testes are situated just behind the middle of the body (·3 mm. behind the ventral sucker). They are symmetrical and lateral. They lie behind the ends of the intestines, but are separated from them by folds of the uterus. The length of each is about ·5 mm. They are fairly thick and elongated, and their outer margin is always distinctly divided into three large lobes, which may in addition be slightly crenated. The inner margins are completely obscured by the overlying folds of the uterus.

The ovary is situated over the right posterior quadrant of the ventral sucker and projects half beyond it. It is obliquely ovoid and measures ·24 × ·17 mm. A large shell-gland lies close to its inner side. The yolk-glands are entirely lateral and of limited extent. They reach from the anterior border of the ventral sucker to about the middle of the testes. Again in this species, however, the posterior limit is extremely variable, and may be anywhere between the anterior and posterior borders of the testes, but never beyond them. The anterior limit is practically constant. The uterus fills almost the whole of the post-acetabular region, but its configuration is entirely different from that in *Lechriorchis*. Here, again, the descending limb is small and almost empty; reaching the posterior end of the body it turns into the ascending limb. In this case, however, accommodation for the enormous number of ova is obtained not by excessive dilatation, but by numerous convolutions, the diameter of the uterus not being very greatly increased. The convolutions have a markedly transverse disposition, extending from side to side of the body. In the region of the testes the convolutions are shorter and stouter. An additional small convolution is formed in front of the ventral sucker. The uterus terminates in a well-marked vagina, which is about a third of the length of the cirrus-pouch. The ova are very like those of *Lechriorchis validus*, but are usually more oval. They have a large distinctly-marked operculum, and

they vary in length from ·034 mm. to ·042 mm. by ·017 mm. to ·021 mm. in breadth. The average is about ·4 × ·2 mm.

In this species, again, no case of amphitypy was observed, and the only pronounced variation was in respect of the posterior limit of the yolk-glands as described above.

The species obviously presents a close resemblance to the genus *Renifer* Pratt, *sens. strict.* In the shortness of the intestinal diverticula, the symmetrical situation of the testes, and the extreme lateral position of the genital aperture, the agreement is complete. The essential difference lies in the configuration of the uterus. *Renifer ellipticus* Pratt, the type species, is unfortunately not fully grown and the ultimate disposition is not apparent. In *R. sauromates* Poirier, the uterus is of the same type as in *Lechriorchis validus*, and if this be taken as characteristic of the genus, then *O. formosum* must be separated from that genus.

It is evident that the three genera *Renifer*, *Lechriorchis*, and *Ochetosoma* are somewhat closely related, and they differ from all the other members of the family Lepodermatidæ in the extreme lateral and forward position of the genital aperture. They evidently form the nucleus of a group, but the extremely profuse variety met with in the family renders it somewhat difficult to divide it into definite subfamilies. Provisionally, however, these three genera may be classified under Pratt's subfamily Reniferinæ. That *Pneumatophilus* Odhn., and *Leptophallus* Lühe, are to be included along with these, as Odhner has indicated (2. p. 56), appears to me somewhat doubtful.

The third form which I have to describe here is one of very great interest. It was obtained from a Diamond Water-snake (*Tropidonotus rhombifer*) from North America. The habitat, unfortunately, is not recorded. It bears a certain resemblance to the foregoing species, and belongs to the family Lepodermatidæ, but it possesses an individuality sufficiently marked to constitute a distinct generic type.

Dasymetra conferta, gen. et sp. n. (Plate XXVIII. figs. 8–10.)

The collection consisted of about a dozen specimens, all of which were mature, and measured 3·5–4·6 mm. in length. The body is elongated, slightly flattened and of fairly uniform breadth. The greatest breadth occurs about the middle and is 1–1·4 mm. The length is therefore about 3½ times the breadth. The cuticle is beset throughout its whole extent by long straight spines. It appears to be somewhat deciduous, and in many specimens is absent from a considerable part of the body, especially towards the posterior end. Several specimens, however, retained the cuticle and spines quite intact.

In a specimen of average length (4·2 mm.) the oral sucker measures ·56 mm. in diameter. It is globular, almost terminal and not very muscular. The ventral sucker is practically of equal size, if anything a trifle less. It is somewhat transversely oval, the dimensions being ·52 × ·57 mm. It is only slightly prominent,

not very muscular, and is situated 1·7 mm. from the anterior end. The neck, therefore, comprises $\frac{2}{5}$ of the body-length.

The alimentary canal is highly developed. It consists of a very short prepharynx, with an enormous pharynx measuring ·28 mm. in diameter. The œsophagus is shorter than the pharynx, being only about ·2 mm. long. It is fairly wide, with well-developed musculature and numerous peri-œsophageal cells. It divides into two very wide diverticula, which extend along the sides of the body to near the posterior end. From the latter they are separated by a loop of the uterus. The ends are slightly inflated and somewhat turned in.

The excretory system has the same general structure as in the two previous species. The main stem of the excretory vesicle divides close behind the shell-gland into two limbs, which extend a short distance in front of the ventral sucker. From the vesicle numerous twigs are given off, which divide and subdivide in the lateral fields to form an intricate anastomosis. The most peculiar feature of the excretory system, however, is the pigmented condition of the excretory tubules, which renders them strikingly conspicuous and marks out their course with great distinctness. This feature renders the species unique amongst the Lepodermatidæ. The pigmentation is due to the excretory granules, which are almost black in colour, and which fill the tubules. Only a few of these are to be met with in the vesicle. A main excretory tubule runs along the greater part of the length on each side of the body, ventral to the intestinal diverticula. In front of the ventral sucker it divides into small branches, one of which runs in to join the vesicle, and another runs forward to the oral sucker. At the posterior end it also divides into several branches. It is impossible to say whether this pigmented condition occurs in life or is a post-mortem appearance, but it was certainly present in all the specimens.

The genital aperture is median, just over the intestinal bifurcation (·3 mm. in front of the ventral sucker). It shows a tendency to be deflected very slightly to the left side. In every specimen the long, thick cirrus was exserted. The cirrus-pouch is short and stout, in some cases being almost globular. Its posterior end lies dorsal to the middle of the ventral sucker, but it may extend beyond this to almost the posterior border of the sucker. The pouch has an external wall composed of very thick longitudinal muscle-fibres, with an inner layer of much smaller circular fibres. It contains a small, slightly-coiled vesicula seminalis, a small bulbous pars prostatica, with numerous prostatic cells, and a long ductus and unarmed cirrus. As already mentioned, the latter was exserted in every case, so that the arrangement depicted in fig. 8 (Pl. XXVIII.) must be regarded as hypothetical. The vesicula, prostate, and cirrus all have a very well-marked layer of longitudinal muscle-fibres.

The testes are situated obliquely, the left being well in front of the right, but not entirely so. The former lies about ·3 mm., and the latter ·8 mm. behind the ventral sucker. They are large ovoid

bodies, with entire margins, and their long axes lie nearly in the longitudinal axis of the body. They measure ·5–·7 mm. in length and ·4–·5 mm. in breadth. They are most remarkable, however, for their great thickness, which is equal to or greater than the breadth. They thus occupy nearly the whole body-thickness, a fact which prevents them being obscured by the uterus. They are separated from each other by the wide ascending limb of the uterus, against which they press, and their outer margins are closely apposed to the intestinal diverticula.

The ovary is situated over the right posterior quadrant of the ventral sucker, a short distance behind the end of the cirrus-pouch. It projects well beyond the sucker, and is transversely oval, measuring ·35 mm. × ·28 mm. It lies close up to the dorsal surface of the body. Close to the inner side of the ovary and a little behind it, lies a large shell-gland, with a short ootype. A receptaculum seminis is absent, but Laurer's canal is present. The yolk-glands are rather voluminous. They are entirely lateral and peripheral, and they extend from the level of the genital aperture, or rather behind it, to near the posterior border of the right testis. Here, again, the anterior limit is fairly constant, but the posterior limit is somewhat variable, less so, however, than in the two species already described. The follicles are large and arranged in regular dendritic groups. All the follicles are connected up by short ducts. They lie close under the surface of the body, extending widely under the dorsal surface, but being much more restricted ventrally, where they do not overlap the inner wall of the intestinal diverticula.

The uterus is large and voluminous, but is not much convoluted. It is intermediate in type between that of *Renifer* and *Ochetosoma*, but resembling the former more than the latter. It consists of a small descending limb, which has a dorsal position and contains a considerable number of ova. This runs to the posterior end of the body where it forms a small convoluted knot, a fairly symmetrical pair of loops, one on each side, being thrown up towards the ends of the intestinal diverticula. From this knot emerges the ascending limb, which rapidly widens but does not attain its maximum width till it has passed in front of the testes. It is never so enormously dilated as in *Lechriorchis validus*. Near the middle of the ventral sucker it runs into a remarkably powerful vagina, which is as long as or somewhat longer than the cirrus-pouch. The vagina possesses unusually muscular walls, there being a very thick layer of longitudinal fibres and an equally thick layer of circular fibres. It is surrounded by a great mass of peri-vaginal cells, and it is lined by a thick layer of cuticle from which small regular cuticular processes extend into the lumen. The ova are numerous, dark brown and oval, with large well-marked operculum. They measure ·033–·037 mm. in length and ·016–·019 mm. in breadth, the usual size being ·036 × ·018 mm.

The genus *Dasymetra* may be defined as follows:—

Lepodermatidæ; with moderately flattened body, entirely covered

with spines. Intestine with large pharynx and wide diverticula which extend near but not quite to the posterior end. Excretory vesicle Y-shaped with numerous side twigs. Genital aperture median, a short distance in front of the ventral sucker. Cirrus-pouch short and plump; vesicula and pars prostatica short; cirrus long. Receptaculum seminis absent; Laurer's canal present. Yolk-glands extensive, dendritic, peripheral. Uterus forming a small convoluted knot at the posterior end of the body, with a wide unconvoluted ascending limb. Vagina long and very muscular. Ova about ·035 mm. long.

Type, *D. conferta*, sp. n.

The type-specimens of these species are deposited in the Museum of the Royal College of Surgeons, London. Co-types at the Zoological Society's Gardens.

References.

(1) M. Braun, 1902.—Fascioliden der Vögel. Zoolog. Jahrbücher; Abt. f. Syst. xvi. pp. 64–67.

(2) T. Odhner, 1910.—Nordostafrikanische Trematoden, grossten-teils vom Weissen Nil. i. Fascioliden. Results of the Swedish Zoological Expedition to Egypt and the White Nile, 1901. No. 23 A, pp. 22–76.

(3) J. Poirier, 1886.—Trematodes nouveaux ou peu connus. Bull. Soc. Philomat. Paris, sér. 7, vol. x. pp. 24–6.

(4) H. S. Pratt, 1903.—Descriptions of Four Distomes. Mark Anniversary Volume, pp. 23–38.

(5) J. Stafford, 1905.—Trematodes from Canadian Vertebrates. Zool. Anzeiger, xxviii. p. 691.

EXPLANATION OF THE PLATES.

The following letters apply to all the figures:—

D.St. Yolk-glands.	*R.S.Ut.* Receptaculum seminis uterinum.
Ex. Excretory vesicle.	*T.*, T_1, T_2. Testes.
Ex.T. Excretory tubules.	*Ut.* Uterus.
J. Intestinal diverticula.	*Vg.* Vagina.
K.St. Ovary.	*V.S.* Vesicula seminalis.
P.Pr. Pars prostatica.	

Plate XXVII.

Lechriorchis validus.

Fig. 1. Young specimen. Ventral view. × 25.
2. Adult specimen. Ventral view. × 20.
3. Transverse section near ends of intestinal diverticula. × 53.
4. Transverse section, immediately in front of ventral sucker. × 50.
5. Ovum. × 500.

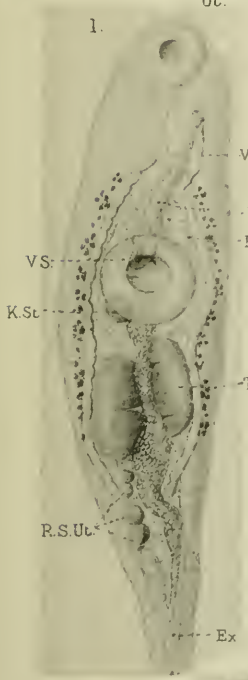
Plate XXVIII.

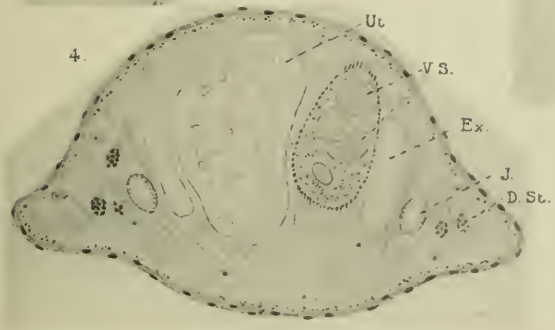
Ochetosoma formosum.

Fig. 6. Ventral view. × 30.
7. Ovum. × 550.

Dasymetra conferta.

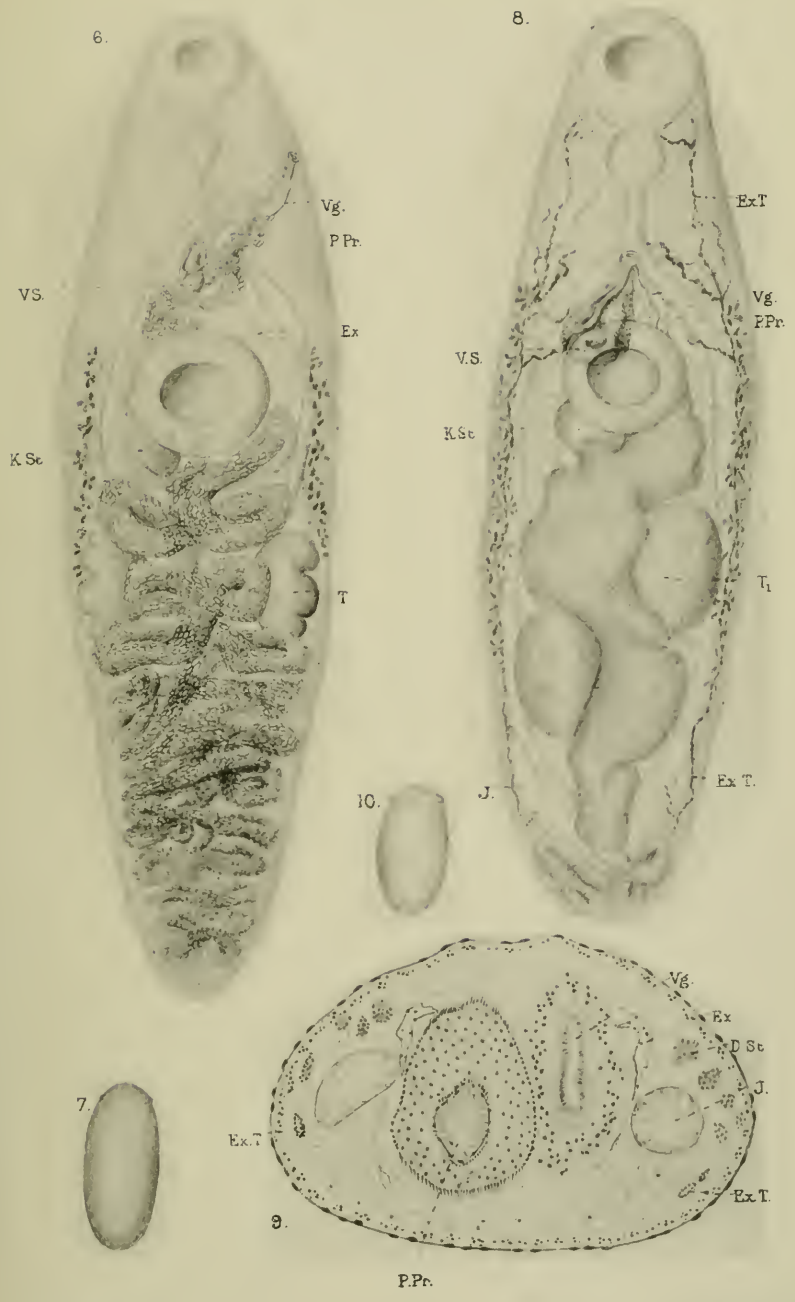
Fig. 8. Ventral view. × 30.
9. Transverse section, a little in front of ventral sucker. × 60.
10. Ovum. × 500.





M. Rhodes. del.

LECHRIORCHIS VALIDUS



M. Rhodes, del.

6. 7. OCHETOSOMA FORMOSUM.
8 - 10. DASYMETRA CONFERTA

[*Reprinted from the* Proceedings of the Royal Society, B. Vol. 84]

The Permeability of the Yeast-Cell.

By Sydney G. Paine, Research Scholar in the Biochemical Department, Lister Institute, London.

(Communicated by Arthur Harden, F.R.S. Received July 12, 1911.)

The question of the permeability of plant membranes and of the protoplasm lining plant cells has received just attention from time to time, the method usually employed being based upon plasmolysis, a phenomenon first described by Nägeli in 1855 (1), and subsequently investigated by Pfeffer (2), De Vries (3), and Overton (4). The results of their experiments tend to show that purely physical diffusion laws cannot always interpret osmotic phenomena as exhibited by living plant cells, but that in some cases there is evidence of specific permeability.

Nathanson (6) finds that the permeability of protoplasm for any substance is not constant, but varies according to the concentration within and without the cells, and he holds that these variations cannot be accounted for in a purely physical manner.

In 1899 Overton (5), in a series of very comprehensive investigations, observed the similarity existing between solutions in oils and in the ectoplasmic layer of the cytoplasm; he showed especially that many substances could be made to enter the plasma by dissolving them in oils, and suggested the hypothesis that the absorption of such substances by living plants might be due to the presence of lecithin and cholesterol in the plasmatic layer. This hypothesis, however, does not account for the semi-permeability exhibited by various plant membranes towards inorganic salts. Again Adrian Brown (7) has shown that the seed coat of *Hordeum vulgare* exhibits a remarkable degree of impenetrability to strong acids and to metallic salts, while it admits of ready diffusion of such substances as alcohol, aldehyde, acetone, iodine, and certain salts of mercury and cadmium. Armstrong (8) has attempted to explain these results on the theory of "hormones," but it appears to the author that strong support to Overton's hypothesis is afforded by these experiments of Adrian Brown. It has been shown by Overton that iodine and mercuric chloride, as well as the above-mentioned organic substances, are readily soluble in cholesterol. Since these include most of the substances which were found by Brown to be capable of entering the barley grain, it seemed advisable to ascertain whether the remaining substances found by him to enter the seed, namely, cadmium iodide and trichloracetic acid, also possessed this property of solubility

in oils. Of these, cadmium iodide is not soluble in oil or cholesterol, but is known to form a double compound with lecithin; trichloracetic acid was found to dissolve with great readiness in both xylene and cholesterol. It would, however, be unfair to assume from these facts that the presence or absence of cholesterol and lecithin in the cells is the determining factor for the diffusion of these substances. It seems to the author more likely that there may be some characteristic of the molecule, or possibly a determinate size of particle, which gives to certain substances the property of solubility both in oils and in living protoplasm.

Diffusion must play a very important part in the technique of cytological investigation, since proper fixation depends largely upon the rapid and uniform diffusion of such agents as mercuric chloride, iodine, osmic and chromic acids, etc. Variations in diffusion capacity may possibly account in large measure for the differences observable under the influence of the several fixing agents. It is conceivable, for instance, that in nuclear investigations the extreme sharpness of definition with which the astral rays and spindle-fibres have been brought out by some workers may be due to inferior fixation; in other words, to a contraction along lines of dynamic activity, wherein the protoplasm has become so altered as to possess a coefficient of penetrability for the special fixing agent employed which is different from that of the surrounding medium.

The primary object of this research has been to investigate the osmotic behaviour of the yeast-cell towards those substances which have been found to influence alcoholic fermentation. Harden and Young (9) have shown that when phosphates, arsenites or arsenates are added to yeast-juice and sugar a considerable increase of the rate of fermentation is produced. When, however, these substances are applied to living yeast it has been found that in almost all cases no such result occurs. Slator (10) has shown that when neutral potassium phosphate is added to living yeast the only effect produced on the initial rate is that of a small inhibition. Iwanoff (11) on the contrary, and more recently Euler and Lundeqvist (17), have demonstrated a small increase in the total fermentation produced by addition of phosphates to living yeast and glucose, but these effects are not comparable with that produced on yeast-juice.

Another phenomenon of a similar character is observed in the case of hexosephosphates, which are freely hydrolysed and fermented by yeast-juice, but are scarcely affected by living yeast. As the formation and decomposition of hexosephosphates plays an essential part in alcoholic fermentation by yeast-juice, it is a matter of great importance to ascertain whether these salts can penetrate the cell.

The Plasmolysis of Yeast by Various Substances.

Preliminary experiments were first made on the degree of plasmolysis of yeast-cells produced by immersion of the cells in solutions of different substances. For this purpose equal weights of pressed brewer's yeast were intimately mixed with equal volumes of the several solutions and allowed to stand for varying intervals of time. Well-mixed samples were then drawn up into capillary tubes of 10 cm. length, which were afterwards sealed at one end and spun simultaneously in a centrifuge. The columns of residue and of the clear liquid were then measured in millimetres, and from these the ratio of the length of the column of residue to that of the whole column (residue + liquid) was calculated.

In all these experiments wort and 7 per cent. alcohol were employed as standards, the effect produced being practically the same.

Table I.

No.	Solution.	Percentage length of column of residue after—		
		2 hrs.	20 hrs.	70 hrs.
22*a*	Wort	63·7	63·5	63·5
b	Water	70·0	62·2	63·4
c	Alcohol, 7 per cent.	60·5	63·3	63·4
d	Sodium chloride, 0·3 molar	59·9	55·2	54·0
e	„ 0·1 „	66·2	61·5	61·0
f	Sodium phosphate, 0·3 molar	63·3	57·1	54·9
g	„ 0·1 „	70·6	63·3	62·5

It was found that when yeast was treated with water the cells at first increased in volume, but later returned to their original state. An initial dilatation also occurred with decimolar solutions of sodium chloride and sodium phosphate, but eventually, in both these cases, a slight amount of plasmolysis was noted. With 0·3 molar concentrations of these substances no increase in volume was observed, and a considerably greater final degree of plasmolysis was produced than was the case with the weaker solutions.

These numbers show, further, that equilibrium is practically established in 20 hours at air temperature, but not in 2 hours.

Adrian Brown (*loc. cit.*) finds that solutions of certain non-electrolytes seem to possess the power of entering the barley grain, whilst others, such as sugar and urea, do not; also that trichloracetic acid, an acid which becomes strongly ionised in dilute solution, enters quite freely. The fact that most of the entering substances are non-electrolytes, he observes,

cannot be taken as an explanation of the diffusion phenomena. A possible solution of the problem has already been advanced (p. 289).

It seemed desirable to ascertain whether these substances would act in a similar manner towards the yeast-cell, it being at first thought that permanent plasmolysis of the cell might be taken as an indication that no diffusion of the dissolved substance into the cell had occurred, an idea which further experiments proved to be untenable (p. 294). The following table contains the results of experiments with acetone, urea, mercuric chloride, cadmium iodide, sulphuric and trichloracetic acids. Since the volumes measured in the narrow tubes were very small, these experiments were made on a larger scale; 50 grm. of pressed yeast were stirred up with 50 c.c. of solutions of the various substances which Adrian Brown found to be of interest. They were allowed to stand for varying lengths of time in the cold room at a temperature ranging from $-2°$ to $+2°$, and were then all spun simultaneously in the centrifuge and the columns of residue and liquid carefully measured. The corresponding volumes were ascertained by gauging the capacity of the vessel.

Table II.

No.	Solute.	Percentage volume of spun residue after—					
		1 hr.	2 hrs.	3 hrs.	4 hrs.	20 hrs.	25 hrs.
176*a*	Sulphuric acid, molar	43·0	41·0	36·5	37·0	36·5	36·5
b	Sodium chloride, molar ...	43·0	43·0	45·0	43·0	40·0	40·0
c	Trichloracetic acid, molar ..	42·0	42·0	37·5	37·5	37·5	37·5
d	Alcohol, 7 p.c. (control) ...	62·0	62·0	58·0	58·0	62·0	60·0
e	Acetone, molar	64·0	62·0	60·0	60·0	64·0	62·0
f	Urea, molar	53·0	57·0	58·0	58·0	61·0	61·0
g	Cadmium iodide, molar ...	43·0	43·0	42·0	42·0	41·0	41·0
h	Mercuric chloride (satd.)...	54·5	50·5	50·5	43·0	38·5	35·5

These results exhibit striking differences when compared with Adrian Brown's experiments. When this observer immersed dried grains of barley in different solutions, water entered as freely and rapidly from solutions of alcohol, acetone, and trichloracetic acid as from pure water, a fact which was interpreted as showing that these substances readily penetrated through the diffusion membrane. In *d* and *e* above alcohol of 7 per cent. and acetone produced no permanent plasmolysis and would seem to diffuse quite readily. Urea also produced no permanent plasmolysis, in striking contrast to Brown's result, where the entrance of water was strongly inhibited. The behaviour of trichloracetic acid also stands in contrast to its behaviour towards the barley grain.

In the experiments with cadmium iodide and mercuric chloride considerable plasmolysis occurred, but this fact cannot be taken to indicate that no diffusion of these substances had taken place, since a marked change was observed in the appearance of the yeast. The cells became much paler in colour and more opaque, while the liquid assumed a dark brownish-grey colour. From the solid appearance of the cells, it would seem that these salts had penetrated through the membrane and coagulated and contracted the cytoplasm; this appears the more evident in the case of the relatively weaker solution of mercuric chloride, where plasmolysis went on slowly up to the end of three hours, after which a strong and rapid contraction took place. These facts are explicable on the assumption that the proteins of the ectoplasm are slowly coagulated during the first three hours and by contraction leave open access for the solution to the inner layers.

In another series of experiments 10 grm. of pressed yeast were weighed out into each of several Nessler glasses and treated with 20 c.c. of the solutions tabulated below. The tubes were allowed to stand in ice water during about 20 hours. They were then centrifuged in batches of four, each batch being spun for exactly 21 minutes. The tubes were then weighed, the liquids poured off into measuring vessels and the weights of the residues ascertained by re-weighing the tubes.

No plasmolysis was produced by solutions of acetone, urea and the lower concentrations of alcohol up to 10 per cent. With the higher concentrations

Table III.

No.	Solute.	Total weight.	Weight of residue.	Percentage of residue.	Volume of liquid poured off.
178, 1	Water	29·8	14·3	48·0	15·5
2	Alcohol, 7 per cent.	29·5	14·0	47·5	15·6
3	„ 10 „	29·3	13·9	47·1	15·5
4	„ 20 „	29·1	13·0	44·7	16·5
5	„ 25 „	29·0	12·6	43·4	17·2
6	„ 30 „	28·7	11·2	39·0	18·3
7	Acetone, molar	29·9	14·7	49·2	15·0
8	Urea, molar	29·9	14·3	47·8	15·5
9	Glycerine, molar	29·8	12·9	43·3	16·5
10	„ $\frac{1}{10}$ molar	29·7	14·6	49·1	15·5
11	Sulphuric acid, $\frac{1}{2}$ molar	30·4	8·7	28·6	21·7
12	Acetic acid, molar	29·8	8·6	28·9	21·0
13	Sodium chloride, molar	30·5	10·5	34·4	19·0
14	„ $\frac{1}{10}$ molar	29·9	14·0	46·8	15·8
15	Sodium acetate, molar	30·4	11·7	38·5	18·0
16	Sodium sulphate, $\frac{1}{2}$ molar	30·9	12·1	39·1	18·0
17	Magnesium sulphate, molar	31·5	11·8	37·5	18·5
18	„ $\frac{1}{10}$ molar	30·9	14·6	47·3	15·0
19	Sodium phosphate, $\frac{3}{10}$ molar	30·4	12·8	42·1	17·4
20	Sodium arsenate, $\frac{2}{10}$ molar	30·4	12·7	41·8	17·5

of the latter plasmolysis was well marked, the effect increasing with increasing concentration. Since no appreciable effect is produced by concentrations up to 10 per cent. it seems possible that diffusion of alcohol is freely permitted. The plasmolysis produced by more concentrated alcohol may be a result of changes in the molecular constitution of the protoplasm. Comparison with the case of mercuric chloride tends to strengthen this view.

The liability to plasmolysis by 20 per cent. alcohol exhibited by different samples of yeast seems to vary with the physical condition of the yeast.

In the experiments given in Table III 10 grm. of pressed yeast were stirred up with 20 c.c. solution, allowed to stand over night in the cold room, and centrifuged next morning.

Table IV.

Solution.	Yeast A.		Yeast B.		Yeast C.		Yeast D.		Yeast E.	
	Volume of liquid poured off.	Weight of residue.	Volume of liquid poured off.	Weight of residue.	Volume of liquid poured off.	Weight of residue.	Volume of liquid poured off.	Weight of residue.	Volume of liquid poured off.	Weight of residue.
Water	14·0	—	15·0	14·8	15·5	14·0	13·7	16·1	15·5	14·3
Alcohol, 5 per cent....	—	—	14·5	14·7	16·0	14·1				
„ 7 „ ...	14·0	—	15·0	14·6	14·5	13·9	14·1	15·7	15·6	14·0
„ 10 „ ...	—	—	14·5	14·5	15·0	14·0	14·0	15·7	15·5	13·9
„ 15 „ ...	15·0	—	14·0	14·5	15·0	14·1				
„ 20 „ ...	17·0	—	15·5	14·0	15·5	14·4	15·0	13·8	16·5	13·0
„ 25 „ ...	—	—	—	—	—	—	16·2	13·1	17·2	12·6
„ 30 „ ...	—	—	—	—	—	—	21·0	9·4	18·3	11·2

Yeast C was an old sample which had been kept in the cold room for about 24 hours after being received from the brewery, and it is worthy of note that no plasmolysis of this yeast by concentrations of alcohol up to 20 per cent. could be detected by this method.

The fact that permanent plasmolysis of yeast is produced by higher concentrations of alcohol, by mercuric and cadmium salts which precipitate the proteins within the cell, and by acids which prevent the activity of the cell, shows that for this cell the existence of permanent plasmolysis is no criterion of the non-diffusibility of the solutions producing it.

In order to arrive at definite results on this subject, it was clearly seen that quantitative estimations of the substance under investigation in the yeast-

cells and in the liquid surrounding the cells would be necessary. Attempts were therefore made to obtain the yeast-cells minus the liquid which normally fills the interstices in an ordinary cake of yeast.

This was eventually accomplished by enclosing the moist yeast cake of the brewery, or yeast obtained as residue after centrifuging, in chain cloth and subjecting it to the pressure of a small hand press. A white friable cake of yeast was obtained which appeared to be composed of dry cells. This was proved to be the actual fact by the following series of experiments:—(1) Total solid estimations in the same pressed cake gave uniform results, showing the cake to be homogeneous. (2) Two pressings of the same yeast-paste gave dry pressed cakes with the same total solid content. (3) Samples of brewery yeast were pressed out and subsequently suspended in the expressed wort, centrifuged and again pressed out, the total solids in the two press cakes were exactly equal. (4) Direct estimations of a salt solution left in the interstices of press cake showed that the greatest volume of liquid thus held by 100 grm. of dry pressed yeast was 0·5 c.c.

Method of Experiment.

The yeast was prepared by pressing out the cake of yeast as received from the press of the brewery, washing being avoided in order to prevent disturbance of the equilibrium of the cell contents. A considerable amount of wort was thus removed. A known weight of this dry yeast was then suspended in a certain volume of the liquid under experiment and allowed to stand for about 20 hours in the cold, after which it was found that osmotic equilibrium between the cells and the solution was attained. The mixture was then centrifuged until the liquid portion was cleared from suspended yeast-cells. The clear fluid was then poured off and the pasty yeast residue was pressed out as described above.

In order to ascertain the weights of yeast-cells and liquid after the experiment, and the distribution of the solute under examination, the following determinations were necessary:—Total solid estimations of the initial and final liquid, and of the initial and final pressed yeast, together with estimations of the dissolved substance in the initial and final liquid, and, in all cases where this was possible, in the initial and final yeast.

The assumption has been made that the total solid matter present in the mixture remains constant during the experiment, an assumption only justified when no loss of carbon dioxide owing to auto-fermentation of the yeast takes place.

Effect of Auto-fermentation.

A small loss of carbon dioxide, accompanied by production of alcohol, does always take place, and the results are subject to error arising from this cause. It was found by direct experiment that, under the conditions employed, a maximum loss of about 0·9 grm. of solid was caused by auto-fermentation.

A loss of 1 grm. in total solids during an experiment has been found to produce a positive error of 5 per cent. on the calculated weight of liquid outside the yeast-cells, so that the results to be given later must be considered to be liable to an error of this order. To eliminate this factor as far as possible, the mixture was allowed to stand in the cold room at a temperature ranging from -2 to $+1°$.

Calculation of the Formula for Obtaining the Weight of Liquid outside the Yeast-cells.

Let l = weight of initial liquid, y = weight of initial yeast, then the total weight $W = l + y$; and if g = percentage of solids in l, and c = percentage of solids in y, then the total solid matter present $V = \frac{lg}{100} + \frac{yc}{100}$.

Both these values W and V are assumed to remain constant during the experiment.

Further, let L = weight of final liquid, Y = weight of final yeast, then $Y = W - L$; and if G = percentage of solids in L, and E = percentage of solids in Y, then the total solid matter $V = \frac{LG}{100} + \frac{YE}{100}$. Substituting for Y in terms of W and L,

$$\frac{LG}{100} + \frac{(W-L)E}{100} = V,$$

whence $L = \frac{EW - 100V}{E - G}$, and Y is obtained by difference from W.

Having thus calculated the weights of liquid and yeast at the end of the experiment, the distribution of the substance under investigation, before and after treatment, is found from the analyses of the initial and final liquid and the initial and final yeast; at the same time, any interchange of other solid matter and of water is made manifest.

Alcohol.

Table V shows the results of experiments with alcohol of various concentrations. This substance was chosen as it might be expected to diffuse freely through the envelope of the cell. Assuming the whole of the water

within the cells to be available for mixture with alcohol, the concentrations of alcohol inside and outside the cells, after osmotic equilibrium had been established, would be equal.

Now the grammes of substance (in this case alcohol) per 100 grm. of water within the yeast (P), divided by the grammes of substance per 100 grm. of water outside the cells (P_1), gives a measure (K) of the amount of diffusion which has taken place. In the case under discussion, therefore, K would be expected to be equal to unity.

In these experiments the increase of alcohol due to auto-fermentation could not be neglected. The total amount of alcohol present at the end of the experiment was therefore determined by analysis of the liquid poured off and of the residue after centrifuging. The weight of alcohol due to auto-fermentation was thus found by difference from the original amount, and was embodied in the calculation for the weight of liquid outside the cells. The formation of an amount of alcohol (F) during an experiment occasions a loss of an approximately equal amount of carbon dioxide to be

Table V.—Diffusion of Alcohol of varying Concentrations.

No.	Conditions.		Yeast.		Liquid.		P.	P_1.	K.
			Initial.	Final.	Initial.	Final.			
75	Alcohol, 2·5 molar, stood 3 hrs. at room temperature	Total ...	100·00	95·70	98·00	101·90			
		Alcohol	4·29	5·80	11·47	10·78	9·91	12·08	0·82
76	„	Total ...	100·00	95·50	98·00	102·30			
		Alcohol	4·50	5·60	11·47	10·42	9·13	10·95	0·83
77	Alcohol, 2·5 molar, stood 20 hrs. in cold room	Total ...	100·00	98·17	98·00	99·57			
		Alcohol	3·14	5·50	11·47	9·50	8·97	10·65	0·84
80	„	Total ...	100·00	91·08	98·00	106·16			
		Alcohol	3·48	5·36	11·47	10·62	9·62	11·30	0·85
100	Alcohol, 1·3 molar, stood 20 hrs. in cold room	Total ...	100·00	97·56	98·80	100·47			
		Alcohol	3·39	3·42	6·00	6·63	5·31	7·13	0·74
101	„	Total ...	100·00	94·01	98·80	104·31			
		Alcohol	3·47	3·55	6·00	6·84	5·95	7·07	0·84
81	Water, stood 20 hrs. in cold room	Total ...	50·00	47·07	100·00	102·59			
		Alcohol	2·39	0·64	—	2·11	1·96	2·11	0·92
85	„	Total ...	100·00	94·16	100·00	105·48			
		Alcohol	4·44	1·37	—	3·42	2·16	3·38	0·64
90	„	Total ...	100·00	99·83	100·00	99·90			
		Alcohol	4·65	1·86	—	3·19	2·66	3·34	0·79

subtracted from the total weight (W), and a loss of approximately twice the amount of solid matter (2F) to be subtracted from V, since the alcohol is formed according to the equation

$$C_6H_{12}O_6 = 2CO_2 + 2C_2H_5OH.$$

If F = weight of alcohol by auto-fermentation, W − F = the total weight at the end of the experiment, and V − 2F = the weight of solid matter finally present, and the formula given on p. 296 becomes

$$L = \frac{E(W-F) - 100(V-2F)}{E-G} = \frac{EW - 100V - F(E-200)}{E-G},$$

and Y is obtained by difference from W − F.

The table shows very clearly that alcohol penetrates freely through the cytoplasm of yeast, but the interesting fact is observed that when equilibrium is established the ratio of alcohol to water is, in every case, less within the cell (P) than it is outside (P_1), and that these ratios stand to one another in a fairly constant proportion (K).

This points to the possibility that some of the water of the protoplasm is bound up in such a manner as to render it unavailable as a solvent for alcohol. This view is supported by the high value for K found in Experiment 81, wherein old yeast was employed which contained a very large vacuolar space and a correspondingly decreased layer of cytoplasm.

The method is specially interesting, as it affords a very clear insight into the interchange of material occurring between the cells and the surrounding liquid. For instance, in Experiment 81 (yeast in water), 0·36 grm. of alcohol have been formed by auto-fermentation within the yeast, bringing the total alcohol up to 2·75 grm. Of this 2·11 grm. have passed out into the surrounding water, leaving 0·64 grm. in the final yeast; 0·70 grm. of solid matter have passed out from the yeast, and 0·73 grm. of solids have been fermented. At the same time there has been an entrance of 0·21 grm. of water into the cells, which is also accounted for as having left the liquid.

Since in these experiments the value of K appeared to be independent of the concentration of the alcohol, it seemed advisable to investigate this further, and also to try the effect of variations in other directions. Since the factor K depends solely upon the analyses of the components of the final system, in each of the experiments about to be described only two estimations of total solids and two of alcohol were necessary.

The results of these experiments are contained in the following Table VI In all cases, except where otherwise stated, the duration of the diffusion was 20 hours at the temperature of the cold room :—

Table VI.—Shewing Diffusion of Alcohol.

No.	Alcohol.	Time of standing.	Yeast.			Liquid.			P.	P_1.	K.
			Solids.	Alcohol.	Water.	Solids.	Alcohol.	Water.			
	per cent.	hrs.	per cent.	per cent.	per cent.	per cent.	per cent.	per cent.			
					Effect of varying Concentration.						
119	20	20	38·63	8·15	53·22	1·52	15·02	83·46	15·31	17·99	0·85
120	10	20	27·95	5·97	66·08	0·83	9·33	89·84	9·04	10·38	0·87
121	5	20	30·49	3·48	66·03	0·91	5·75	93·34	5·27	6·16	0·85
122	0	20	28·71	1·72	69·57	0·92	2·94	96·14	2·47	2·98	0·83
					Effect of varying Time of Standing.						
131	20	17	33·00	7·67	59·33	0·69	13·67	85·64	12·93	15·96	0·81
132	20	41	34·49	8·14	57·37	0·74	13·90	85·36	14·19	16·28	0·87
133	20	65	34·99	7·74	57·27	1·03	13·80	85·17	13·52	16·20	0·83
134	20	89	33·46	9·88	56·66	1·32	13·87	84·81	13·85	16·35	0·85
					In Auto-fermented Yeast.						
81	0	20	29·26	1·37	69·37	0·69	2·06	97·25	1·96	2·11	0·92
139	10	20	30·82	4·87	64·31	1·29	8·27	90·44	7·57	9·14	0·83
144	10	20	30·76	6·06	63·18	4·07	9·40	86·53	9·59	10·86	0·88
					Effect of varying Quantity of Liquid.						
	Yeast.	Alcohol, 10 per cent.									
141	50 grm.	50 cc.	34·50	4·11	61·39	0·95	7·73	91·32	6·69	8·46	0·79
142	50 „	100 „	34·61	4·35	61·04	0·47	7·86	91·67	7·13	8·57	0·83
143	50 „	200 „	34·88	4·10	61·02	0·27	8·05	91·68	6·72	8·78	0·76

In this table, the factor K is found to be remarkably uniform, and to be uninfluenced by variations in the conditions. It is further noteworthy that, under the action of concentrations ranging from 0 to 10 per cent. (Experiments 119–122), no marked variation in the solid content of the yeast is produced. This stands in further confirmation of the results obtained during the earlier experiments on plasmolysis given in Table III, p. 293, in which it is shown that the plasmolysing effect of alcohol is inappreciable until a concentration of 20 per cent. is employed. Again, as was previously observed, the extent of plasmolysis by the higher concentration is not uniform in different samples of yeast; for instance, in the yeast of Experiment 119, an increase of 10 per cent. in the total solids is observed, while in the yeast of similar experiments the increase was only 1·8 and 4·7 per cent. It was noticed also that, with this concentration, a change had taken place within

the cell, making it impossible to obtain the usual white friable press cake, but rather a brown-coloured and putty-like mass.

This change may possibly take the form of a contraction of the cytoplasm of the cell, and, since the depth of the layer of cytoplasm varies under different conditions of the yeast, the amount of plasmolysis sustained by a given sample of yeast when immersed in 20 per cent. alcohol may possibly be determined by the relative proportions of the cell occupied by the cytoplasm and by the vacuole.

In Experiments 131–134 a gradual diffusion of solid matter from the yeast into the surrounding liquid is observed to have taken place; this is probably due to the production of diffusible solids by autolysis of the cell contents.

In Experiments 81 and 139 yeast was employed which had been kept in the cold room for three days. The yeast of 144 was a sample which had been dried in the air at room temperature. The original percentage of total solids in this dry yeast was 68·4 per cent. On immersion in 10 per cent. alcohol the yeast absorbed liquid very quickly, and eventually, after 20 hours, 50 grm. had increased to about 110 grm. Notwithstanding this large influx of liquid, the diffusion factor is seen to be not widely different from the normal.

Sodium Chloride.

This substance was taken as being a typical salt dissociated into its ions more or less completely in dilute solution. Four experiments were made, the results of which are given below. The salt was estimated in the liquid by the method of Carius, the amount in the yeast being calculated by difference. Preliminary experiments had shown that when 50 grm. of pressed yeast

Table VII.—Diffusion of M/10 Sodium Chloride.

No.	Conditions.		Liquid.		Yeast.		P.	P_1.	K.
			Initial.	Final.	Initial.	Final.			
60	Allowed to stand 20 hrs. in cold store	Total ...	100·50	101·00	50·00	49·50			
		NaCl ...	0·58	0·54	nil	0·04	0·11	0·54	0·21
61	Allowed to stand 20 hrs. in cold store	Total ...	100·50	100·30	50·00	50·20			
		NaCl ...	0·58	0·55	nil	0·03	0·08	0·55	0·15
64	Allowed to stand 3 hrs. at room temperature	Total ...	100·50	104·30	50·00	46·20			
		NaCl ...	0·58	0·58	nil	nil	—	0·56	—
65	Allowed to stand 3 hrs. at room temperature	Total ...	100·50	101·60	50·00	48·90			
		NaCl ...	0·58	0·58	nil	nil	—	0·58	—

were suspended in distilled water and the mixture centrifuged, only a faint trace of milkiness was produced by the addition of acid silver nitrate to the liquid poured off.

These results show that the diffusion of sodium chloride is slow. A definite quantity of substance enters the cell when yeast is suspended in M/10 sodium chloride solution and allowed to stand over night, although no diffusion is noticed after a suspension of three hours only.

Ammonium Sulphate.

The next experiments were made with ammonium sulphate, as being a substance which is of service to the yeast, and which is a sufficient source of nitrogen in artificial culture. In these experiments two concentrations of the solution have been employed, of approximately one-tenth and three-tenths molar respectively.

Table VIII.

No.	Conditions.		Liquid.		Yeast.		P.	P_1.	K.
			Initial.	Final.	Initial.	Final.			
67	Stood 20 hrs. in cold store. Solution, 0·1 molar	Total	100·40	97·84	50·00	52·56			
		$(NH_4)_2SO_4$	1·31	1·21	nil	0·10	0·28	1·26	0·22
68	Stood 20 hrs. in cold store. Solution, 0·1 molar	Total	100·50	99·21	50·00	51·29			
		$(NH_4)_2SO_4$	1·31	1·19	ni	0·12	0·34	1·22	0·28
69	Stood 3 hrs. in cold store. Solution 0·1 molar	Total	100·60	97·30	100·00	103·30			
		$(NH_4)_2SO_4$	1·29	1·23	nil	0·06	0·09	1·28	0·07
70	Stood 3 hrs. at room temperature. Solution, 0·1 molar	Total	100·60	99·48	50·00	51·12			
		$(NH_4)_2SO_4$	1·31	1·21	nil	0·10	0·28	1·24	0·23
71	Stood 3 hrs. at room temperature. Solution, 0·1 molar	Total	100·60	102·50	50·00	48·10			
		$(NH_4)_2SO_4$	1·31	1·28	nil	0·03	0·09	1·27	0·07
145	Stood 20 hrs. in cold store. Solution, 0·1 molar. Air-dried yeast	Total	90·10	58·78	30·00	61·32			
		$(NH_4)_2SO_4$	1·14	0·98	nil	0·16	0·37	1·76	0·22
149	Stood 20 hrs. in cold store. Solution, 0·3 molar	Total	50·50	58·87	50·00	41·63			
		$(NH_4)_2SO_4$	1·84	1·71	nil	0·13	0·47	3·00	0·15
150	Stood 20 hrs. in cold store. Solution, 0·3 molar. Air-dried yeast	Total	100·00	90·86	50·00	59·14			
		$(NH_4)_2SO_4$	3·65	3·40	nil	0·25	0·61	3·91	0·16
151	Stood 20 hrs. in cold store. Solution, 0·3 molar. Air-dried yeast	Total	100·00	75·44	50·00	74·56			
		$(NH_4)_2SO_4$	3·65	3·13	nil	0·52	1·03	4·41	0·23

The ammonium sulphate was estimated in the initial and final liquids only, the amount in the final yeast being found by difference.

The results with ammonium sulphate are found to be very similar to those obtained with sodium chloride.

Experiments 145 and 151 are specially interesting as the initial yeast in these cases contained a large percentage of solid matter. In the former, where one-tenth molar ammonium sulphate was employed, 33·17 grm. of water have entered the yeast while only 0·16 grm. of the salt have been carried in, and in the latter from three-tenths molar solution 25·47 grm. of water and 0·52 grm. of salt have entered. The ratio of the concentration inside the cells to the concentration outside is the same in both cases.

The rate at which a sample of air-dried yeast will absorb water and recover turgescence is very remarkable. In No. 145 when 30 grm. of the yeast were mixed with 60 c.c. of solution such a stiff paste was obtained, within two minutes, that it could only be stirred with difficulty. It is further worthy of note that yeast which has been dried in air returns to its normal condition of turgescence when immersed in water, as shown by the percentage of total solids. In all samples of fresh pressed yeast the total solid content has been found to vary from 28 to 35 per cent. Total solid estimations of three air-dried samples gave 68·4, 50·5, and 37·5 per cent.; when immersed in water these yeasts became turgid with a normal solid content of 30·5, 31, and 32·4 per cent. respectively.

The envelope of such dried and shrivelled cells, though readily permeable by water, does not admit of the entrance of a 1/10 molar solution of ammonium sulphate, but selects from it a large quantity of water and only a relatively small quantity of the salt. The liquid surrounding the cells therefore becomes considerably concentrated. In Experiment 145, for instance, the concentrations of salt in the initial and final liquids were 1·26 and 1·66 respectively.

Copper Sulphate.

A peculiar resistance to the entrance of copper salts is exhibited by the protoplasm of *Penicillium glaucum* (12), growth of which has been found possible on a medium containing as much as 21 per cent. copper sulphate, although very much smaller quantities down to 3 per cent. have occasionally proved destructive. In view of this result it seemed advisable to investigate the effect of solutions containing copper upon the protoplasm of the yeast-cell. 100 grm. of yeast were suspended in 100 grm. 1/10 molar $CuSO_4$, allowed to stand for the usual time in the cold room, and the distribution of the copper determined.

Table IX.

Initial liquid.		Initial yeast.		Final liquid.		Final yeast.	
Weight.	$CuSO_4$.	Weight.	$CuSO_4$.	Weight.	$CuSO_4$.	Weight.	$CuSO_4$.
grm. 100	grm. 0·78	grm. 100	grm. —	grm. 128·4	grm. 0·36	grm. 71·6	grm. 0·43

A remarkable degree of plasmolysis was observed and the shrunken cells were of a pale green colour and solid appearance, the cytoplasm had evidently entered into combination with the $CuSO_4$ and had been precipitated thereby, so that the factor K in this case is much greater than unity, namely, 3·36. When this yeast was added to sugar solution no fermentation was produced.

Sodium Phosphate.

These experiments have been made with solutions of the di-sodium salt of two concentrations, roughly 1/10 and 3/10 molar, and with water.

Table X.

No.	Conditions.		Yeast.		Liquid.	
			Initial.	Final.	Initial.	Final.
103	Liquid containing 1·42 grm./100 c.c. Na_2HPO_4, stood 20 hrs. in cold store	Total	50·00	50·34	50·50	50·16
		P_2O_5	0·75	0·76	0·35	0·38
104	,, ,,	Total	50·00	49·91	50·50	50·59
		P_2O_5	0·81	0·87	0·35	0·36
107	,, ,,	Total	100·00	99·90	101·30	101·40
		P_2O_5	1·63	1·64	0·71	0·71
109	,, ,,	Total	100·00	100·74	101·50	100·76
		P_2O_5	1·39	1·55	0·71	0·63
111	Liquid containing 4·16 grm./100 c.c. Na_2HPO_4	Total	100·00	95·50	104·00	108·50
		P_2O_5	1·63	1·97	2·08	1·80
112	,, ,,	Total	100·00	96·60	104·00	107·40
		P_2O_5	1·59	2·01	2·08	1·80
105	Water	Total	100·00	102·02	100·00	97·98
		P_2O_5	1·43	1·49	—	trace
108	Water	Total	50·00	53·16	50·00	46·84
		P_2O_5	0·81	0·80	—	0·01
114	Water	Total	100·00	107·80	100·00	92·20
		P_2O_5	1·53	1·56	—	trace

In the estimations, the organic matter was destroyed by Neumann's method and the phosphoric acid was precipitated with magnesium citrate mixture.

With the weaker concentration, which was found to be isotonic with yeast, no exchange of phosphoric acid took place, but from a solution containing 4 per cent. of sodium phosphate, approximately 0·3 molar, entrance of phosphoric acid into the cells was well marked.

Sodium Hexosephosphate.

For the purpose of this research hexosephosphate was of all substances of greatest interest, since Harden and Young (13) have found that hexosephosphoric acid is continually being built up and broken down again in the fermentation of sugar by yeast-juice. When they added this substance to living yeast, however, no evidence of its fermentation could be obtained. It was of special importance, therefore, to determine whether any of the substance had been able to diffuse into the yeast-cells.

The solution of hexosephosphoric acid was prepared by the method described by Young (14) and was neutralised to litmus with caustic soda. Four concentrations of the salt have been employed and the results are given in the following table. In each case the time of standing was 20 hours at a temperature between −2 and 0°.

Table XI.—Diffusion of Sodium Hexosephosphate.

No.	Conditions.		Yeast.		Liquid.	
			Initial.	Final.	Initial.	Final.
169	Concentration, 0·035 molar = 0·14 normal. Standing 20 hrs. in cold room	Total.....	100·00	89·80	100·00	110·20
		P_2O_5	1·42	1·36	0·50	0·51
174	Concentration, 0·06 molar = 0·24 normal	Total.....	100·00	102·50	100·00	97·50
		P_2O_5	1·42	1·55	0·84	0·80
172	Concentration, 0·126 molar = 0·504 normal	Total.....	100·00	92·90	100·00	107·10
		P_2O_5	1·26	1·52	1·80	1·56
177	Concentration, 0·23 molar = 0·93 normal	Total.....	100·00	92·66	100·00	107·34
		P_2O_5	1·66	1·99	3·33	2·98

The results are strikingly similar to those obtained with sodium phosphate. Where the concentration was small, as, for instance, in Experiment 169, no definite entrance of phosphorus took place; with higher concentrations, however, the increase of P_2O_5 in the yeast became well marked. Experiment 177 showed that 0·33 grm. of P_2O_5, equal to 2·5 c.c. of molar solution

and to 1/10 of the total amount in the liquid, have been transferred from the liquid to the yeast. The influence of this solution upon the fermentation of yeast was studied according to the method described in a preliminary communication by Harden and Paine (15), and, although the initial rate of auto-fermentation was increased, the total volume of gas yielded was not greater than that given by a water control, and, moreover, the rate of auto-fermentation produced was exactly comparable to the rate under the influence of sodium phosphate of the same normality. It would seem from this that, although this substance is capable of entering the yeast-cell, it is not able to penetrate through to the sphere of activity of the hydrolysing enzyme.

Sodium Arsenate.

Sodium arsenate was specially interesting, since Harden and Young (16) have found that solutions of arsenates have an enhancing influence on the rate of fermentation of sugar by yeast-juice.

The following table gives results of three experiments. The estimations were made by digesting the yeast and liquid with nitric and sulphuric acids, and, after dispelling the nitric acid, reducing the arsenic acid with hydriodic acid. The liberated iodine was removed by titration with thio-sulphate and the arsenious acid precipitated with sulphuretted hydrogen, collected on a tared filter, washed successively with water, alcohol and carbon bisulphide, dried at 100° and weighed. The results are expressed in terms of anhydrous sodium arsenate.

Table XII.—Diffusion of Sodium Arsenate.

No.	Conditions.		Yeast.		Liquid.		P.	P_1.	K.
			Initial.	Final.	Initial.	Final.			
164	Concentration, 2·02 per cent. = 0·11 molar. Stood 20 hrs. in cold room	Total ...	100·00	102·40	100·00	97·60			
		Arsenate	nil	0·15	2·02	1·86	0·22	1·95	0·11
165	„ „	Total ...	100·00	97·90	100·00	102·10			
		Arsenate	nil	0·26	2·02	2·10	0·39	2·12	0·18
166	Concentration, 3·35 per cent. = 0·18 molar	Total ...	100·00	88·20	100·00	111·80			
		Arsenate	nil	0·28	3·35	3·27	0·49	3·03	0·16

These results are essentially similar to those obtained with sodium chloride and ammonium sulphate, the factor K shows a fair degree of uniformity and indicates definite but very imperfect diffusion of the substance.

Summary and Conclusions.

The early experiments on plasmolysis of yeast seemed to indicate that the envelope of the yeast-cell was impermeable by inorganic salts generally while it allowed of the ready diffusion of such substances as alcohol, acetone, and urea, which have been known to pass with ease through many forms of living protoplasm.

Quantitative estimations have shown the power of diffusion of alcohol to be very different from that of inorganic salts. On immersion of yeast in dilute alcohol, varying from 5 per cent. to 20 per cent., the ratio of the concentration within the cells to that of the liquid outside becomes practically constant, and independent of the absolute concentration. Alcohol is believed to diffuse quite readily into the cell, but at the same time this ratio is not unity, but a constant which deviates only slightly from 0·85. Probably the whole of the water in the cell, which is removed by drying at 98° C., is not available for diffusion of alcohol. The amount of water thus bound up, possibly as a constituent of the protoplasmic complex, appears to vary somewhat at different stages in the life-history of the cell, but the method was not considered sufficiently delicate to render further study of this interesting phenomenon advisable in this way.

All salts which have been tried have been taken up by yeast from moderately concentrated solutions, and in the cases of sodium chloride and ammonium sulphate even from dilute solutions. But, whereas with alcohol the amount entering the yeast during three hours was practically equal to the amount which entered on prolonged immersion, with these salts the process was a slow one. After three hours no sodium chloride had entered from a decimolar solution, and considerably less ammonium sulphate was found in the yeast than was the case after longer standing. From decimolar solution of sodium phosphate no entrance of phosphorus was appreciable even after 20 hours' standing, but from more concentrated solution, 0·3 molar, a well marked entrance was observed. Since phosphates are essential for the life of the yeast and are gradually assimilated and accumulated from very dilute solutions, the envelope must admit the necessary amount of these substances required by the cell for its metabolism. The amount thus absorbed during the time of these experiments would naturally be very small and indeterminable.

With regard to the entrance of salts, which the experiments have shown to occur, the following considerations are of interest. Since the yeast must of necessity be analysed as a whole, the question as to how far into the cells the various substances have penetrated must, at present, remain in doubt. While most salts do show some entrance into the cells, the factor which is taken as an expression of permeability is, except in the case of copper

sulphate, comparatively small (0·1—0·25 as against 0·85 in the case of alcohol). It seems very probable that the apparent entrance of salts is a result of adsorption in the surface layers of the cell rather than absorption, or it may be that the salt particles are kept back by a differential septum according to the hypothesis of H. E. Armstrong (8), and that they remain in the interstices of such membrane.

The experiments with hexosephosphate are particularly interesting in this connection, since this substance is present in yeast and is readily hydrolysed and fermented by yeast-juice. The fact that when this substance is added to yeast there is no evidence whatever of its being fermented would seem to indicate that it had not been able to penetrate through to the seat of fermentative activity. It thus seems highly probable that the apparent entrance of this salt, which is well marked, is merely a surface phenomenon.

In conclusion, the author desires to express his best thanks to Dr. A. Harden, at whose instigation the work was commenced, and whose kindly interest and numerous suggestions have been highly esteemed.

REFERENCES.

1. Nägeli. 1855. 'Pflanzenphys. Unters.,' vol. 1, p. 21.
2. Pfeffer, W. 1877. 'Osmotische Untersuchungen,' Leipzig.
„ 1886. 'Unters. aus d. Bot. Inst. Tübingen,' vol. 2. p. 179.
„ 1890. "Plasmahaut und Vakuolen," 'Abh. Math.-Phys. Kl. Sächs. Gesell.,' vol. 16, p. 187.
3. De Vries, H. 1877. 'Die Mechan. Ursachen d. Zellstreckung.'
„ 1884. "Methode zur Analyse d. Turgorkraft," 'Jahrb. f. Wiss. Bot.,' vol. 14, p. 427.
„ 1884 A. 'Bot. Zeit.,' vol. 46, p. 229.
„ 1888 B. *Ibid.*, vol. 46, p. 393.
„ 1889. *Ibid.*, vol. 47, p. 309.
4. Overton. 1895. 'Vierteljahrsschr. d. Naturf.-Gesell. Zürich.'
5. „ 1899. *Ibid.*
6. Nathanson. 1902. 'Jahrb. f. Wiss. Bot.,' vol. 38, p. 241.
7. Brown, Adrian J. 1909. 'Roy. Soc. Proc.,' B, vol. 81, p. 82.
8. Armstrong, H. E. 1909. *Ibid.*, B, vol. 81, p. 94.
9. Harden and Young. 1908. *Ibid.*, B, vol. 80, p. 299.
10. Slator. 1908. 'Chem. Soc. Trans.,' vol. 93, p. 217.
11. Iwanoff. 1910. 'Bio-chem. Zeit.,' vol. 25, p. 171.
12. Pulst, quoted by Pfeffer, 'Physiology of Plants,' Eng. trans., vol. 2, p. 260.
13. Harden and Young. 1908. 'Roy. Soc. Proc.,' B, vol. 80, p. 299.
14. Young. 1909. *Ibid.*, B, vol. 81, p. 528.
15. Harden and Paine. 1911. 'Chem. Soc. Proc.,' vol. 27, p. 103.
16. Harden and Young. 1911. 'Roy. Soc. Proc.,' B, vol. 83, p. 451.
17. Euler and Lundeqvist. 1911. 'Zeitschrift f. physiol. Chemie,' vol. 72, p. 97.

Harrison and Sons, Printers in Ordinary to His Majesty, St. Martin's Lane.

[*Reprinted from the Journal of Physiology,*
Vol. XLII. Nos. 5 *&* 6, *July* 15, 1911.]

ON THE QUESTION OF THE IDENTITY OF PEPSIN AND RENNET.

By AGNES ELLEN PORTER,
Lister Institute Research Scholar.

(*From the Lister Institute of Preventive Medicine, London.*)

The question of whether the activities ascribed to rennet and pepsin are in reality due to one and the same ferment, has been now under discussion for some ten years without any clear solution being arrived at. This continued difference of opinion is, according to a recent writer—A. Rakoczy—due to the fact that most supporters of the identity theory have used stomach extracts other than those in which true rennet has been recorded. The suggestion that the two ferments were identical was first put forward by Pawlow (1901), who has since that time found many supporters. Among these are Pawlow and Parastschuk (1904, p. 451), Sawjalow (1905, p. 20), Nencki and Sieber (1901, p. 312), Michaelis (1909, p. 234), Van Dam (1910, p. 336). All these authors believe that one and the same ferment acts as rennet in neutral solution and as pepsin when the reaction is acid. According to Michaelis pepsin is amphoelectric and only when carrying a positive charge, as in acid solutions, is peptic. He suggests that in neutral solutions when it carries a negative charge it may functionate as rennet.

On the other hand Hammarsten (1910, p. 158) and Rakoczy (1910, p. 460) regard the two ferments as independent. They base their belief upon the want of proportion between these activities in different stomach extracts and commercial preparations. Rakoczy has been able to effect separation of the rennet from the peptic functions by dialysis of stomach extracts whereby the pepsin was precipitated and the rennet left in solution (p. 444). He also found that weaker solutions of hydrochloric acid would extract rennet from the stomach wall, while stronger solutions extracted pepsin (p. 453).

Those who believe the ferments to be identical do so because they find the functions running parallel under various treatments. The disappearance of rennet only, which takes place when a rennet-pepsin preparation is warmed for a few days, has been explained by Van Dam (p. 322). This is not due to disappearance but merely to masking of the rennet by the appearance of substances inhibitory to coagulation but not to digestion. These could be got rid of by dialysis, leaving pepsin and rennet behind in their original proportions. Funk and Niemann (1910, p. 272) find an entire parallelism between the ferments on filtration. Spiro (1906, p. 368) and Petry (1906, p. 355) regard rennet as proteolytic, but only to casein.

There are several points about this theory of the identity of pepsin and rennet which at first sight must strike everyone as remarkable. The actions seem so very different and indeed opposite. How can the same ferment both precipitate and dissolve the same protein? If precipitation were necessarily the first step, and by altering the concentration of ferment or salts, or the surface of the zymolyte, made the second step of digestion possible, this difficulty would be lessened. The process is, however, not so simple. Coagulation may occur without digestion, digestion without coagulation, or both may occur. It has indeed been stated by Zuntz and Sternberg (1900, p. 362) and by Hawk (1904, p. 46), that milk protein which has been coagulated by rennet is less easily digested than fresh milk protein.

It is generally supposed that inhibitory substances act by forming a complex with and masking the ferment itself. This has been shown for the anti-pepsin and anti-trypsin of Weinland, and for serum anti-rennet, by Weinland (1902, p. 52), Dastre and Stassano (1903, p. 634), and Fuld and Spiro (1900). On the other hand these inhibitory substances are acknowledged to be specific for each function (if not for each ferment). Authors who believe that pepsin and rennet are identical, *e.g.* Van Dam, have even explained a want of parallelism under treatment, by the appearance of inhibitory substances, hindering the one action while leaving the other unchecked. These statements appear so contradictory that in order to treat the identity theory with justice, it is necessary to leave on one side for the moment the possibility that inhibitory substances, such as amino-acids, unite with the ferment, but to regard them as acting in some other way, *i.e.* on the substrate. This is necessary because the specificity of these inhibitory substances is so very much more obvious than their mode of action on the ferment. For example, we have Van Dam's adventitious rennet-inhibitory sub-

stances which did not act on pepsin, there is also the strong rennet preparation, which I have described (1910, p. 385) as anti-peptic.

I was led to go into this question because I had come across this rennet preparation just mentioned, which was actively milk-coagulating and at the same time strongly anti-peptic. This circumstance appeared at first peculiar enough to merit further investigation. With this in view, I obtained several commercial rennet and pepsin preparations. These were:

Mackenzie's Rennet, 1909 and 1910 (Edinburgh), liquids,
Melmoth's Rennet, date unknown, liquid,
Evans, Lescher and Webb's Rennet, date unknown, liquid,
Byk's Rennet powder,
Ray's Pepsin powder.

Method. Peptic activity was tested by means of Mett's tubes containing coagulated serum, egg-white, or milk. Where milk was used, the ferment was mixed with the milk before it was drawn up into the capillary tubes. It was then allowed to set at 37°, when it could be cut into short lengths, and placed in 0·25 % HCl. Serum was most often used, as it is so quickly acted on. Two small lengths of serum were always placed in the ferment, and the four eaten ends measured with a fine metal ruler, or with the vernier of a sliding stage under the low power of a microscope. The four counts were added together to give the units of digestion.

This method is not without error, but its extreme rapidity, simplicity, and the fact that digestion and inhibition are so marked and striking to the eye, make it very serviceable. The chief objection to the method, namely that peptic action is lessened by products of digestion gathering in the capillary tube and preventing the diffusion of fresh pepsin to the ever retreating surface of protein, is of less consequence in this instance.

Sörensen's method (1908, p. 45) of testing for the increase of neutral carboxylic groups in proximity to amino groups, by means of titration with neutral formalin-phenolphthalein solution, was discarded, after trial, as unsuitable. After days of digestion the increase in this acidity was too slight to cover the margin of error sufficiently.

Other methods of testing for increasing albumoses depend upon methods of separation by precipitation which are unsatisfactory.

The dissolving action of the pepsin, which is so rapid at the first step, is so very much slower in the lower stages of digestion, that by

measuring the first stage as with Mett's tubes the most satisfactory and obvious results can be obtained.

Measurement with edestin (Fuld and Levison, 1907, p. 473) was used only to estimate peptic action, for demonstrating inhibition it was unfortunately useless.

Anti-peptic action of rennet preparations.

In the following experiments an attempt has been made to discover whether the other rennets, like Mackenzie's, were anti-peptic, if the anti-pepsin were accompanied by evidences of the presence of amino-acids, if it could be got rid of by means of dialysis, and if, on its removal, pepsin could recover power in proportion to the rennet present. All four liquid rennets were strongly anti-peptic, the rennet power hardly so at all.

(1) *Mackenzie's Rennet*, 1909.

Rennet action on 3 c.c. milk = 1/4000, + 0·2 c.c. 10 % $CaCl_2$ = 1/17000.

Peptic action, in 24 hours, on edestin—none visible.

" " " fibrin "

" " " egg-white "

" " " casein "

" " " serum—very slight.

Reaction with methylorange, 1 c.c. required 0·05 c.c. N/10 HCl.

" " phenolphthalein per 1 c.c. required 0·25 c.c. N/10 KOH.

" " neutral formalin-phenolphthalein solution per c.c. required 0·63 c.c. N/10 baryta.

Anti-peptic action.

No. of exp.	Units of serum digested by: 0·5 c.c. pepsin 0·5 c.c. water — 0·125 % HCl	0·5 c.c. pepsin 0·5 c.c. water — 0·18 % HCl	0·5 c.c. pepsin 0·5 c.c. rennet — 0·125 % HCl	0·5 c.c. pepsin 0·5 c.c. rennet — 0·18 % HCl	Peptic power reduced to	
1	26	—	8·7	—	33·4 %	
2	19	—	5·2	—	27·3	
3	79	—	40·5	—	51·3	
4	24	—	12·5	—	52	
5	34	—	14	—	41·1	
6	20	—	2	—	10	
7	46	—	8	—	17·2	
8	21	—	7	—	33·3	
9	—	66	—	38	—	57·5
Averages:	...	...	...	...	33·2	57·5

N.B. The pepsin used in the above as in later experiments was Ray's pepsin powder 1 % in HCl of the above strengths. The rennet was regarded as neutral.

The amount of acidity in this rennet after the addition of formalin-phenolphthalein solution, and even with phenolphthalein alone, is suggestive of a fair amount of protein and amino-acid impurities. The rennet was dialysed in parchment for two days against running water. After this treatment it was neutral with phenolphthalein, and after the addition of formalin solution, required per c.c. only 0·066 c.c. N/10 baryta to neutralise. It had lost at the same time all its anti-peptic power.

Table illustrating loss of anti-peptic power in Mackenzie 1909 *dialysed against water.*

	Units of serum digested by			
	0·5 c.c. pepsin +0·5 c.c. water		0·5 c.c. pepsin +0·5 c.c. Mackenzie 1909 dialysed	
No. of exp.	0·125 % HCl	0·18 % HCl	0·125 % HCl	0·18 % HCl
1	21	—	18·6	—
2	—	10	—	8
3	—	89	—	85
4	—	92	—	99
5	—	66	—	73
6	—	104	—	109
7	—	83	—	76
Averages		66·4		66·9

Although this dialysed rennet had retained all its milk-coagulating properties in full measure, it had gained no peptic activity whatever, and even on serum it was totally inactive. This same dialysed rennet turned after ten days spontaneously inactive as rennet. This point will be dealt with later more fully.

The organic acidity in this rennet (given below under *Reaction*) points to a fair amount of protein and amino-acid impurities. After dialysis against running water for two days, the milk-coagulating action of this rennet was reduced from 1/340000 (with $CaCl_2$) to 1/42500 (with $CaCl_2$), which was still powerful. The anti-peptic action had disappeared with the organic acidity, but had left *no peptic action behind.* The preparation contained in fact, after this purification, much rennet but no pepsin. Dialysed against N/20 HCl without extra $CaCl_2$ the rennet coagulated milk at dilutions of 1/1100 as rapidly as in its original state. (The limits of its rennet action were unfortunately not tested.) Organic acidity and anti-peptic had disappeared. Peptic action was not absent, but had not increased.

(2) *Mackenzie's Rennet*, 1910.

Rennet action on 3 c.c. milk = 1/8000, + 0·2 c.c. 10 % $CaCl_2$ = 1/340000.

Peptic action, in 24 hours, on edestin—none visible.

,, ,, ,, egg-white ,,

,, ,, ,, casein ,,

,, ,, ,, serum—slight.

Reaction with methylorange, 1 c.c. required 0·05 c.c. N/10 HCl.

,, ,, phenolphthalein per c.c. required 0·265 c.c. N/10 KOH.

,, ,, neutral formalin-phenolphthalein solution per c.c. required 0·572 c.c. N/10 baryta.

Anti-peptic action.

No. of exp.	Units of serum digested by: 0·5 c.c. pepsin 0·5 c.c. water — 0·125 % HCl	0·5 c.c. pepsin 0·5 c.c. water — 0·18 % HCl	0·5 c.c. pepsin 0·5 c.c. rennet — 0·125 % HCl	0·5 c.c. pepsin 0·5 c.c. rennet — 0·18 % HCl	Peptic power reduced to	
1	19	—	5·8	—	30·5 %	
2	62	—	14	—	22·5	
3	28	—	8	—	28·5	
4	103	—	28	—	27·1	
5	47	—	20	—	44	
6	—	89	—	45	—	50·5 %
7	—	92	—	58	—	63
8	—	66	—	38	—	57·5
9	—	104	—	56	—	53·8
Averages :	...	...	...	...	30·6	56·2

Table showing digestive activity of Mackenzie Rennet 1910 *dialysed against* N/20 HCl, *unaffected.*

No. of exp.	Units of serum digested by: 0·5 c.c. Original Mackenzie 1910 0·5 c.c. N/10 HCl	0·5 c.c. Same dialysed against N/20 HCl + 0·5 c.c. N/20 HCl
1	29	29
2	36	34
3	32	25
4	31	31

Table showing want of inhibition by dialysed Mackenzie 1910.

No. of exp.	Units of serum digested by: 0·5 c.c. pepsin + 0·5 c.c. acid — 0·21 % HCl	0·5 c.c. pepsin + 0·5 c.c. acid — 0·27 % HCl	0·5 c.c. pepsin 0·5 c.c. 1910 dialysed against N/20 HCl — 0·21 % HCl	0·5 c.c. pepsin 0·5 c.c. 1910 dialysed against N/20 HCl — 0·27 % HCl
1	32	—	42	—
2	123	—	110	—
3	—	65	—	73
4	—	105	—	110
Averages :	81·25		83·75	

A companion sac containing 20 c.c. of this rennet was placed in 30 centimetres of N/30 HCl. Only a trace of rennet could be demonstrated in this HCl solution in contact with the sac, it became however rapidly distinctly anti-peptic.

Table showing that the anti-pepsin had passed through the sac.

	Units of serum digested by			
	0·5 c.c. pepsin +0·5 c.c. water		0·5 c.c. pepsin +0·5 c.c. fluid outside sac containing Mackenzie 1910	
No. of exp.	0·125 % HCl	0·18 % HCl	About 0·125 % HCl	0·18 % HCl
1	28	—	16	—
2	103	—	72	—
3	—	89	—	72
4	—	92	—	80
Averages:	80		60	

(3) *Melmoth's Rennet.*

Rennet action on 3 c.c. milk = 1/800, + 0·2 c.c. 10 % CaCl = 1/17000.

Peptic action, in 24 hours, on egg-white—none visible.

" " " serum "

" " " casein "

" " " edestin—some action.

Reaction – methylorange, 1 c.c. required 0·2 c.c. N/10 HCl.

– phenolphthalein per c.c. required 0·31 c.c. N/10 KOH.

– neutral formalin-phenolphthalein solution per c.c. required 1·15 c.c. N/10 baryta.

Anti-peptic action.

	Units of serum digested				
	0·5 c.c. pepsin 0·5 c.c. water		0·5 c.c. pepsin 0·5 c.c. rennet		
No. of exp.	0·125 % HCl	0·18 % HCl	0·125 % HCl	0·18 % HCl	Peptic power reduced to
1	24	—	0	—	0
2	21	—	0	—	0
3	47	—	0	—	0
4	—	89	—	7	7·8 %
5	—	92	—	6	6·5
6	—	66	—	4	6
7	—	104	—	8	7·7

The acidity in this rennet after the addition of neutral formalin-phenolphthalein solution, and even with phenolphthalein alone, indicates a large amount of protein and amino-acid impurities. Unlike the Mackenzie rennets it contained salts, especially NaCl, in a concentration sufficient to account for inhibition without regard to organic impurities. Dialysis against N/20 HCl for two days sufficed to rid the rennet almost completely of anti-peptic ingredients. The milk-coagulating power was unaffected by this treatment, while a certain amount of peptic power was gained, equal to that of Mackenzie 1910 on serum, but slight in comparison to that of 1 % pepsin powder.

	Units of serum digested by		
	0·5 c.c. 1 % pepsin powder 0·5 c.c. acid	0·5 c.c. Mackenzie untreated 0·5 c.c. acid	0·5 c.c. Melmoth dialysed 0·5 c.c. acid
No. of exp.	Total acidity...0·18 % HCl	0·18 % HCl	0·18 % HCl
1	89	29	20
2	92	36	30
3	65	22	20

Table showing the loss of anti-peptic power in dialysed Melmoth's Rennet.

No. of exp.	0·5 c.c. pepsin 0·5 c.c. acid 0·18 % HCl	0·5 c.c. pepsin 0·5 c.c. dialysed Melmoth's rennet 0·18 % HCl
1	92	77
2	65	65
3	105	95

A companion sac, containing this rennet, was placed in a few cubic centimetres of N/20 HCl. After two days, not a trace of rennet could be demonstrated in this HCl solution outside the sac, it became however rapidly anti-peptic, reducing the value of pepsin powder to about 36·4 % of its former worth (average of three results) in two days.

Table showing anti-peptic action of HCl *solution in contact with sac containing Melmoth's Rennet.*

	Units of serum digested by		
No. of exp.	0·5 c.c. pepsin +0·5 c.c. water 0·18 % HCl	0·5 c.c. pepsin +0·5 c.c. fluid outside sac containing Melmoth About 0·18 % HCl	Peptic power reduced to
1	89	28	—
2	92	23	—
3	66	39	—
Averages :	82·3	30	36·4

Evans, Lescher and Webb's Rennet.

Rennet action on 3 c.c. milk = 1/4000, + 0·2 c.c. 10 % CaCl = 1/17000 (incomplete).

Peptic action, in 24 hours, on egg-white—none visible.

,, ,, ,, serum ,,

,, ,, ,, casein ,,

,, ,, ,, edestin—slight.

Reaction + methylorange 1 c.c. = 0·1 c.c. N/10 HCl.

+ phenolphthalein (titration difficult) per c.c. = circa 0·37 c.c. N/10 KOH.

Anti-peptic power.

Where equal quantities of 1 % pepsin powder and this rennet were added together there was a total inhibition of peptic activity. This action was sufficiently explained by the presence of a large quantity of salts, especially NaCl ($CaCl_2$ being also present). After dialysis

against N/20 HCl results were similar to those obtained by dialysing Melmoth's rennet. The inhibitory action was much reduced, without much recovery of pepsin taking place.

Byk's Rennet powder 1 %.

Rennet action on 3 c.c. milk = 1/40000, + 0·2 c.c. 10 % CaCl = 1/85000.

Peptic action, in 24 hours, on egg-white—none visible.

" " " serum "

" " " edestin "

Reaction + methylorange 1 c.c. = 0·05 c.c. N/10 HCl (neutral).

+ phenolphthalein per c.c. = 0·005 c.c. N/10 KOH (neutral).

+ neutral formalin-phenolphthalein solution per c.c. = 0·01 c.c. N/10 baryta (neutral).

Anti-peptic action very slight.

	Units of serum digested by			
	0·5 c.c. pepsin + 0·5 c.c. water		0·5 c.c. pepsin + 0·5 c.c. "Byk" 1 % in water	
No. of exp.	0·125 % HCl	0·18 % HCl	0·125 % HCl	0·18 % HCl
1	21	—	19·8	—
2	—	89	—	74
3	—	92	—	66
4	—	66	—	56
5	—	104	—	98
6	—	84	—	65
Average fall to	...	...	...	83 %

This preparation, which dissolved instantaneously in water, appeared to contain no appreciable protein or amino-acid impurities to account for the remarkable want of peptic power in so active a rennet. A 1 % solution in 0·18 % HCl (N/20 HCl) was dialysed against N/20 HCl for two days, gaining thereby absolutely no peptic power whatever. If any anti-peptic action could be ascribed to this rennet before dialysis, certainly after dialysis it appeared wholly indifferent in the matter of digestion, while retaining its remarkably active rennet properties as before.

Table showing indifference of dialysed Byk's Rennet to pepsin.

	Units of serum digested by	
	0·5 c.c. pepsin 0·5 c.c. acid	0·5 c.c. pepsin 0·5 c.c. dialysed Byk's rennet
No. of exp.	0·27 % HCl	0·27 % HCl
1	65	63
2	105	110
3	84	81
Averages :	84·66	84·66

A comparison between this active rennet powder and an active pepsin powder, *i.e.* Ray's, is instructive.

Ray's Pepsin powder 1 %.

Rennet action on 3 c.c. milk = 1/5500 alone, + 0·2 c.c. 10 % CaCl = 1/42500 (strong, but weaker than Byk).

Peptic action on edestin, to 1000 (1 inch long).

,, ,, serum, capillary tube eaten out in a few hours in presence of 0·25 % HCl.

,, ,, casein, capillary tubes 1 inch long eaten out in three hours in presence of 0·25 % HCl (very strong action in comparison with negative action of Byk's rennet).

Reaction – methylorange 1 c.c. – 0·075 c.c. N/10 HCl.

– phenolphthalein per c.c. – 0·15 c.c. N/10 baryta.

– neutral formalin-phenolphthalein solution per c.c. – 0·33 c.c. N/10 baryta.

The above indicates the presence of protein impurities, not present in Byk's rennet.

These rennet and peptic preparations which have been examined display no parallelism whatever in milk-coagulating and peptic power. This want of parallelism is not to be explained by the presence of amino-acids and protein impurities. When purified by dialysis, of all anti-peptic substances, these rennets recovered very little, or no peptic power, while retaining their milk-coagulating properties.

There remains to be described an interesting phenomenon illustrative in another way of the entire independence of these two ferments, rennet and pepsin, in my hands.

A non-anti-peptic rennet-zymoid.

It has been mentioned before that Mackenzie's rennet 1909, dialysed for two days against running water, was immediately after removal from the dialysing sac as active a rennet as before, but after ten days under toluene, was found to have become spontaneously inactive. It had become not only inactive, but inhibitory to fresh rennet. It will be remembered that Mackenzie's rennet 1909 was anti-peptic in its original state. This anti-pepsin had however disappeared during dialysis, the preparation remaining, after dialysis was completed, permanently indifferent to the action of pepsin. Seven estimations gave an average of 66·4 units for 0·5 c.c. pepsin + 0·5 c.c. water, and 66·9 for 0·5 c.c. pepsin and 5 c.c. dialysed "rennet" ("rennet" which was now inactive, and inhibitory to fresh rennet).

This rennet inhibition appearing suddenly in a dialysed preparation, washed clear of amino-acids and unchanged in salt content since the conclusion of dialysis, seems difficult to explain. Van Dam (p. 322)

has mentioned that dialysed rennet is unstable, but he does not state that it may become actually inhibitory.

In order to demonstrate the inhibition an excess of the inhibitory rennet was necessary. This however was to be expected in the case of a ferment which acts so rapidly, and in such small traces, as does rennet. The inhibition was peculiar in that, when exerted against weak or diluted rennets, the curd was not only delayed, but was usually incomplete, a piece of curd floating in liquid milk.

Table showing the rennet-inhibitory action of this dialysed rennet (inactive "1909").

Fresh milk—2 c.c.	Dilution of rennet in milk	Minutes at 40° 8	14	20	25	55
0·2 c.c. Mackenz. 1910 1/50	—	—	—	—	—	—
0·2 c.c. water	1/600	curd	—	—	—	—
0·2 c.c. Mackenz. 1910 1/50	—	—	incom-	—	—	—
0·2 c.c. inactive "1909" ...	1/600	—	plete	—	—	—
0·2 c.c. Melmoth rennet 1/10	—	—	—	—	—	—
0·2 c.c. water	1/120	—	—	curd	—	—
0·2 c.c. Melmoth 1/10 ...	—	—	—	—	—	—
0·2 c.c. inactive "1909" ...	1/120	—	—	—	incom-	—
					plete	
0·2 c.c. Byk rennet 1/500 ...	—	—	—	—	incom-	—
0·2 c.c. water	1/6000	—	—	—	plete	—
0·2 c.c. Byk 1/500 ...	—	—	—	—	—	very in-
0·2 c.c. inactive "1909" ...	1/6000	—	—	—	—	complete

On another occasion, with freshly diluted Byk's rennet:

Fresh milk—2 c.c.	Dilution in milk	Mins. at 40° 18	Hours 2
0·2 c.c. Byk's rennet 1/1000	—	—	—
0·2 c.c. water	1/12000	curd	—
0·2 c.c. Byk 1/1000 ...	—	—	—
0·2 c.c. inactive "1909" ...	1/12000	—	Nil

The absence of protein impurities in the dialysed rennet "1909" made it difficult to explain the appearance of an anti-rennet otherwise than by supposing that the ferment had degenerated into a zymoid form. It must be remembered that zymoid forms of rennet are known to occur. Bearn and Cramer (1907, p. 174) have observed them in heated rennets, and Korschun (1902, p. 375) through binding experiments with serum anti-rennet. The nature of the inhibition exerted

by this dialysed inactive "1909" was such as to support the view that it was due to a zymoid. The fact that only a part of the milk usually coagulated, one small curd floating in the unchanged milk, was suggestive of zymoid formation. While no other dialysed rennet became inhibitory, all deteriorated slightly in value, as did also other highly diluted rennets. Such deterioration was accompanied in high dilutions by this incomplete coagulation, as well as delayed action, probably a tendency to zymoid formation.

As this zymoid was quite indifferent to pepsin, it becomes still more difficult to believe that pepsin and rennet can be identical.

Summary.

Several commercial rennet preparations have been found actively milk-curdling, but anti-peptic.

This anti-peptic influence was due to substances indifferent to rennet. They could not therefore be regarded as acting against the pepsin by uniting directly with it, unless the ferments were independent.

These substances could be easily dialysed away, leaving little or no increase in peptic action.

A rennet powder, and several dialysed rennet preparations, were non-peptic, and non-anti-peptic, while being strongly milk-curdling.

A rennet zymoid occurred spontaneously, which was indifferent to pepsin.

These results are in accord with those of Hammarsten, and speak clearly for the independence of the two ferments, rennet and pepsin.

I desire to express my thanks to Dr Ledingham, Dr Harden and Dr Casimir Funk for kind advice and criticism.

REFERENCES.

Bearn and Cramer. Biochem. Journ. II. p. 174. 1907.
Cramer and Bearn. Proc. Physiol. Soc. p. 36. 1906. This Journal.
Dastre et Stassano. Compt. rend. Soc. de Biol. LV. p. 633. 1903.
Van Dam. Ztsch. f. physiol. Chem. LXIV. p. 316. 1910.
Fuld u. Levison. Biochem. Ztsch. VI. p. 473. 1907.
Fuld u. Spiro. Ztsch. f. physiol. Chem. XXXI. p. 132. 1900.
Funk u. Niemann. Ztsch. f. physiol. Chem. XXXVI. p. 263. 1910.
Hammarsten. Ztsch. f. physiol. Chem. LXVIII. p. 119. 1910.
Hawk. Amer. Journ. Physiol. p. 37. 1904.
Korschun. Ztsch. f. physiol. Chem. XXXVII. p. 366. 1902.

Michaelis. Biochem. Ztsch. xvii. p. 231. 1909.
Nencki u. Sieber. Ztsch. f. physiol. Chem. xxxii. p. 291. 1901.
Pawlow (1901). Quoted from Pawlow u. Parastschuk (1904).
Pawlow u. Parastschuk. Ztsch. f. physiol. Chem. xlii. p. 415. 1904.
Petry. Hofmeister's Beitr. viii. p. 339. 1906.
Porter. Quart. Journ. exp. Physiol. iii. p. 375. 1910.
Rakoczy. Ztsch. f. physiol. Chem. lxviii. p. 421. 1910.
Sawjalow. Ztsch. f. physiol. Chem. xlvi. p. 20. 1905.
Sorensen. Biochem. Ztsch. vii. p. 45. 1908.
Spiro. Hofmeister's Beitr. viii. p. 365. 1906.
Weinland. Ztsch. f. Biol. xliv. p. 45. 1902–3.
Zuntz u. Sternberg. Arch. Anat. u. Physiol. p. 362. 1900.

The Division of the Collar-Cells of the Calcarea Heterocœla.

By
Muriel Robertson, M.A.

With Plate 19.

Introduction.

In 1910 Prof. Minchin and I described (5) the division of the collar-cells in the sponge Clathrina coriacea (Montagu) with a view to obtaining an insight into the behaviour of the basal granule of the flagellum during cell-division. In Clathrina coriacea and the Clathrinidæ generally the nucleus lies at the base of the collar-cells, and, just before division, comes to the apex, which is the position it occupies in the embryo. In the Leucosolenidæ and most Heterocœla, on the other hand, the nucleus lies towards the apex thoughout the whole life of the sponge, and the flagellum arises from a granule which is only very little removed from the nucleus, and attached to it by a double rhizoplast. At Prof. Minchin's suggestion I have recently investigated the division of the collar-cells of two members of the Heterocœla, namely, Grantia compressa and Sycon sp.,[1] to see how the process compares in the two families.

It was at first expected that the basal granule or blepharophast of the flagellum in Grantia and Sycon would lie actually in the nucleus, but that is not the case; the blepharoplast in the vegetative condition of the cell is placed at the apical margin, and is connected with the nucleus by a

[1] Either Sycon ciliatum or S. coronatum; it was not possible to determine the species, since the methods of preservation used were such as dissolved the spicules.

double rhizoplast (figs. 1–5, 21). One is therefore here, as in Clathrina, dealing with an extra-nuclear structure. Both in the earlier paper published with Prof. Minchin and in the present account the work has been done with the view of elucidating the blepharoplast-centrosome question, and not from the standpoint of the morphology of the sponge.

The literature of the subject was fully discussed in the earlier paper, and I propose only to consider here a few additional publications which have particular bearing on the questions under investigation. These will be treated under the "General considerations" at the end of the paper.

Material.

The material was obtained from the Marine Laboratory at Plymouth, and was fixed in various ways. Corrosive sublimate and acetic acid, Flemming's fluid and Bouin's fluid were all used; of these Bouin's fluid gave unquestionably the best result. The stains used were Heidenhain's iron-hæmatoxylin; iron hæmatein, according to Dr. Seidlin's method ('Parasitology,' vol. iv, p. 94), and Twort's combination of neutral red and Lichtgrün; these all gave good results, and were used with good effect to supplement and control each other.

The collar-cells of these sponges form, as is well known, a single layer of epithelium lining the radial tubes or flagellated chambers. The shape of the cell is subject to considerable variation, according to the exact condition of the sponge, and, indeed of the different parts of the sponge, at the moment of fixation; they are, however, usually either flask-shaped (fig. 1), or shaped like chimneys (fig. 2). The collar is a delicate tubular structure which projects from the free end of the cells, and varies somewhat in length according as the cell is fully extended or not. These structures are of extreme delicacy, and are easily destroyed by unsuitable fixation.

The cytoplasm of the cell is granular and vacuolated, and may show large inclusions. The nucleus lies a little below the

apex, and though usually spherical, may sometimes be slightly drawn out towards the top of the cell. The blepharoplast is a small granule staining intensely with iron-hæmatoxylin; with iron-hæmatein it shows quite sharply, but is pale grey in colour, while the chromatin of the nucleus is a blueish-black; with Twort's stain it is not very readily visible, but whenever it can be made out it is always of the cytoplasmic colour, namely, green (fig. 28). There is no shred of evidence in the staining reaction to suggest that the structure in question is of a chromatic or nuclear nature. To insist on the achromatic nature of a centrosome (as in effect the blepharoplast of a collar-cell really is) at the present date seems a useless waste of energy and almost an anachronism, were it not for the recent theories of such well-known workers as Hartmann and Prowazek (**2a**). The centrosome-blepharoplast is situated at the extreme upper edge of the protoplasmic body, and is connected, as has already been said, with the nucleus by a double rhizoplast. In many cases the double nature of this last structure escapes observation, the two strands lying very closely side by side. It is, however, obvious from a careful study of a sufficiently large number of cells that two strands (sometimes widely separated from each other and forming a triangle as in figs. 3 and 5) and not one only connect the blepharoplast with the nucleus. This rhizoplast persists throughout the whole life of the cell except, as will be seen hereafter, for a very short period immediately before division. From the blepharoplast arises the flagellum, which is of considerable length and extends beyond the collar.

To talk of the nucleus of any living and functioning cell as being in the resting state is a self-contradictory phrase which cytologists are only gradually abandoning. In the collar-cells the variety of appearance in the nucleus of the non-dividing cells is particularly striking. Unfortunately from the nature of the case one is unable to correlate the physiological state and the particular appearance of the nucleus, and of the distribution of the chromatin, etc., within

it. One or two points can, however, be observed. It has been noticed that the nucleus immediately before division always shows a dense staining karyosome which disappears during the formation of the primitive spireme (fig. 9). There is, of course, no proof of the converse, and it by no means follows that because the nucleus shows a dense karyosome it is therefore about to enter upon division. In the work on Clathrina a corresponding point was observed, namely, that cells about to divide showed a pale nucleus containing a dense karyosome. Another point in the life of the cell can, as it were, also be caught, and that is the period immediately after division. The young daughter-cells have certain characteristic features to be noted later, and can readily be recognised. The nucleus in these cells is always of the reticulate type, and does not show the karyosome (fig. 21). Whether this behaviour on the part of the karyosome may prove of importance when we know more of what induces a cell to divide cannot be determined at present, but is worth recording in passing.

Mitosis.

The first alteration to be observed in a collar-cell about to divide is usually the disappearance of the flagellum and the subsequent division of the blepharoplast, which has at this stage lost its connection with the nucleus. The exact sequence of these early processes is, however, subject to a good deal of variation, and sometimes the flagellum is retained until after the division of the blepharoplast (fig. 10). In the nucleus itself very characteristic changes take place, culminating in a curious phase which is of very constant occurrence and which corresponds to the spireme-stage. The dense karyosome gradually breaks up (figs. 3–10), and there are formed a number of masses of chromatin which become increasingly definite in appearance; they are connected together at this stage by delicate filamentous strands which do not take up the chromatin stains. This condition corresponds to the spireme-stage, which never reaches a

greater development than this in the case of collar-cells; a glance at figs. 6 and 10 will make it clear how far removed it is in appearance from the long continuous coiled thread so universally seen in more developed types of karyokinesis. Speaking generally, the blepharoplast divides just before the above changes take place in the nucleus, but here again slight variations in the time-relations are very often to be observed. The blepharoplasts gradually move apart, but may sometimes be joined for a time by a strand; this stage is of rare occurrence and is illustrated in fig. 8. More often the blepharoplast divides and the daughter-blepharoplasts move apart without the junction between them persisting. These two little granules are destined to play the part of centrosomes in the coming mitosis, but do not show the radiations passing out from them which are so characteristic of most centrosomes. As time goes on they come to lie on either side of the nucleus, and a spindle-apparatus is formed between them. In fig. 11 an interesting stage is shown where the spireme is not yet quite complete and the first signs of the spindle can be seen arising between the centrosomes on either side of the nucleus. The mitosis proceeds and the equatorial plate is formed, but the chromosomes are not very distinct. At this stage there grows out from the centrosomes on either side the first rudiment of the new flagella (figs. 12–16 and 21), thus exactly repeating the state of affairs observed in Clathrina coriacea. The diaster-stage (figs. 17–19) follows in due course, and the two poles of the spindle each with its mass of chromatin curve upwards through approximately a right angle (figs. 20 and 21).

The centrosome-blepharoplast is still connected with the chromatin mass by a double strand (figs. 20, 21), which is the remains of the spindle. This connection persists as the double rhizoplast already noted and only disappears again just before the next division. In Clathrina this junction disappears and the blepharoplast is completely cut adrift from the nucleus during the vegetative life of the cell. The

two dense chromatin-masses of the diaster-stage re-form into the two nuclei of the daughter-cells. The spireme is entirely suppressed at this point, and the reconstituted nuclei are, as has already been noted, of the reticulate type (figs. 22, 23). The young cells are narrower and project forward beyond the epithelium, and the nuclei appear for a time to be rather smaller than those of the neighbouring cells. The collar disintegrates at an early stage and is re-formed anew in each of the daughter-cells. It is interesting to note in passing that, although this is a quite typical metazoan mitosis, it reveals one or two rather primitive features, such as the very slight development of the spireme, the absence of rays from the centrosomes, and the general indistinctness of the chromosome.

General Considerations.

The main feature of interest in the foregoing account is that we have here another instance of a blepharoplast playing the part of centrosome, and the chief importance of the observation lies in the evidence it brings as to the simple achromatic nature of this structure. In a recent paper Hartmann and Chagas (1) describe the division of a number of free-living flagellates, and some of the forms they deal with are of particular interest.

The part of their work that bears most immediately on the question under consideration is that which treats of the division of Spongomonas uvella and Spongomonas splendida. Here two flagella are present in each individual, and there are two blepharoplasts which are generally not connected in any way with the nucleus and resemble in this particular the condition found in the collar-cells of Clathrina coriacea. The authors are inclined to think that this is due to a secondary absorption of the rhizoplast, and is, as it were, a late condition in development. They are led to this conclusion by the fact that immediately after division the cells of Spongomonas show a quite clear connection between the blepharoplast and the karyosome of the nucleus.

In these forms there is a centriole present in the karyosome. At division the centriole divides into two parts joined by a centrodesmose, and a fairly well-developed mitotic spindle is formed with the centrioles at the two poles and the chromatin arranged in an equatorial plate at the centre. The centrioles generally divide a second time during the course of mitosis, the two granules thus formed lying close beside each other in each case. The behaviour of the flagella is of particular interest; the old flagella are thrown off very early at the outset of division. In the majority of cases the new flagella arise after the completion of mitosis by what the authors call the heteropolar division of the reconstructed karyosomes. It is best to quote the description of the subsequent events in the authors own words: "Da schon bei der Mitose Doppelcentriolen vorhanden waren, sind zwei Möglichkeiten denkbar; entweder sind dieselben bei dieser heteropolaren Teilung einfach verteilt worden, wobei der eine Pol (Basalkörper) zur Bildung des Diplosoms sekundär eine zweite Teilung erfahren musste; oder aber die doppelten Centriole teilen sich gleichzeitig und bilden so direkt das Diplosom. Das zurückgebliebene Doppelcentriol würde dann im Ruhestadium des Kernes infolge fester Aneinanderlagerung scheinbar als einfaches Centriol erscheinen. Bei beiden Möglichkeiten stimmt der Modus der Geisselentstehung prinzipiell mit dem von Cercomonas überein. Daneben kommt aber noch eine sehr interessanter zweiter Modus vor, indem schon im Stadium der Æquatorialplatte die neuen Tochtergeisseln von den Centriolen aus gebildet werden" (pp. 81, 82).

The above description is of great interest and importance, and shows very clearly the centrosomic origin of the blepharoplast in the Protozoa under discussion. I should like, however, to point out that it is an unfortunate confusion of language for the authors repeatedly to talk of heteropolar mitosis of the karyosome when what they both figure and describe is the division of the centriole contained in the karyosome. The word "karyosome" as used in protozoolo-

gical literature means a structure composed of chromatin embedded in an achromatic substance, and which usually contains a centriole or centrosome. In general cytological writing the term is applied to a condensation of chromatin as distinguished from a true nucleolus. In neither sense are the authors justified in saying that the process they describe is really a heteropolar mitosis of the karyosome. The term "mitosis" implies some kind of a partition of all the substance of the structure involved, and heteropolar mitosis of the karyosome means that the plastin, chromatin, etc., have undergone an unequal division.

What these authors show in their excellent work on the two species of Spongiomonas and on Cercomonas parva described in the same paper is the splitting-off of a minute centrosome-like granule which is bound permanently (as in Cercomonas), or for a time (as in Spongiomonas), to the karyosome by a slender thread. The centriolar nature of these granules is abundantly demonstrated in the last sentence of the paragraph quoted above. It is clear that this process is essentially the same as that described in the collar-cells, and brings the blepharoplast of these Protozoa into line with those of the sponge-cells.

The main points raised in all this work are very clearly and broadly put in a valuable article (3) by Hertwig, who comes to the conclusion that the cytoplasm is a compound substance composed (A) of a substance very closely akin and practically identical with the achromatic contents of the nucleus, and (B) of a substance akin to chromatin, and from which this latter is built up. According to Hertwig's idea, the chromatic and achromatic substances are distinct and separated out from each other in the nucleus, while in the cytoplasm the same two substances are present in some sort of combination. The centrosome is for Hertwig simply "ein individualisiertes Stückchen achromatischer Kernsubstanz," and adds that he is prepared to admit that centrosomes may arise outside the nucleus from the achromatic substance of the cytoplasm. These conceptions of Hertwig's are of course to be considered as a

broad fundamental theory rather than as a complete explanation of all the facts. They have, however, the great merit of being formulated from physiological as well as morphological observations, and therefore pay due regard to the processes of cell-life. Very probably the cytoplasmic substance must be regarded as much more complex than is suggested in Hertwig's survey. It is interesting to note in this connection that the work of Reichenow (**4**) has shown that the substance called volutin, which arises in the cytoplasm, is a stage in the building up of the nuclear chromation—or to put the matter more precisely, the chromatin in the nucleus increases at the expense of the volutin in the cytoplasm. The point in this description of Hertwig's view that I wish to emphasise is that it brings out very clearly the achromatic nature of the centrosome. In a later part of the same article he goes on to say that "die Centrosomen und die Basalkörperchen von Wimpern, Geisseln und Pseudopodien analoge Gebilde sind." The proposition embodied in the last sentence has received additional proof and has been further extended by practically all the recent work upon the subject.

Lister Institute,
May, 1911.

Literature.

1. Hartman and Chagas.—"Flagellaten-studien," 'Memorias do Instituto Oswaldo Cruz.,' Tomo 2, Facic. 1, April, 1901.

2a. Hartmann and Prowazek.—"Blepharoplast, Caryosom, und Centrosom," 'Arch. f. Protistenkunde,' Bd. x, 1907.

3. Hertwig.—"Die Protozoen und die Zelltheorie," 'Arch. f. Protistenkunde, Bd. i, 1910.

4. Reichenow.—"Hæmogregarina stepanowi," 'Arch. f. Protistenkunde, Bd. xx, Heft 3, 1901.

5. Robertson and Minchin.—"The Division of the Collar-cells of Clathrina coriacea," 'Quart. Journ. Micr. Sci.,' vol. 55, part 4, November, 1910.

EXPLANATION OF PLATE 19,

Illustrating Miss Muriel Robertson's paper on "The Division of the Collar-cells of the Calcarea Heterocœla."

[The figures are drawn with the aid of the camera lucida at a uniform magnification of 2000 linear.[1]]

Fig. 1.—Collar-cells of Grantia showing the flask shape. Note the karyosome in the nucleus.

Fig. 2.—Collar-cells of Grantia showing the elongated chimney shape.

Fig. 3.—Collar-cell with reticulate nucleus. It also shows the double rhizoplast forming the connection between the blepharoplast and the nucleus (Grantia).

Fig. 4.—Collar-cell of Sycon showing pale nucleus with deeply staining karyosome.

Fig. 5.—Collar-cell of Grantia showing reticulate nucleus with karyosome. Note the double rhizoplast.

Fig. 6.—Early spireme-stage (Grantia).

Fig. 7.—Stage very like that shown in fig. 6, but the spireme is in a still earlier condition (Grantia).

Fig. 8.—Precocious division of the blepharoplast; the specimen is fixed with Flemming's solution (Grantia).

Fig. 9.—Early stage in division; the blepharoplast is newly divided, and the spireme is just being formed at the expense of the karyosome. The flagellum has been thrown off (Sycon).

Fig. 10.—Slightly later stage; the blepharoplast has divided but the flagellum is still retained (Sycon).

Fig. 11.—Slightly aberrant stage; the blepharoplast - centrosomes occupy either end of the spindle which is just forming, while the spireme-stage has not yet been completed (Grantia).

Fig. 12.—Equatorial plate-stage with the chromosomes just splitting. The first rudiment of the new flagellum is visible at one pole (Grantia).

Figs. 13 and 14.—Equatorial plate-stages (Sycon).

Fig. 15.—Equatorial plate showing flagellum growing out from either centrosome (Sycon).

[1] I am indebted to Miss Mabel Rhodes for the care and skill with which she has executed the figures.

Fig. 16.—Equatorial plate showing remnant of collar (Grantia; Flemming fixation).

Fig. 17.—Very early diaster-stage (Grantia).

Figs. 18 and 19.—Diaster-stages (Grantia and Sycon).

Figs. 20 and 21.—Division of cells. Note the junction between the nuclei in course of reconstruction and the blepharoplasts (Grantia).

Figs. 22 and 23.—Newly divided cells. Note the condition of the nucleus. Both the cells and the nuclei are below the normal size (Sycon).

Figs. 24–27.—Stage of division stained with Twort's stain. Note the faint green colour of the blepharoplast-centrosomes (Grantia).

Fig. 28.—Vegetative stage stained with Twort's stain (Grantia).

DIVISION OF COLLAR-CELLS

[*Reprinted from the 'Annals of Tropical Medicine and Parasitology,' Vol. VI, No. 1 B, May, 1912*]

THE DEVELOPMENT OF A LEUCOCYTOZOON OF GUINEA-PIGS*

BY

EDWARD HALFORD ROSS, M.R.C.S. (ENGLAND), L.R.C.P. (LONDON)

(*Received for publication* 1 *April*, 1912)

PLATE VI

The presence of 'bodies' within the large mononuclear leucocytes of guinea-pigs was first noticed by Kurloff (1898). He described them as inclusions; for in a drop of guinea-pig's blood he noted that many of the large lymphocytes contained, within their cytoplasm, clear, spherical vacuoles which were distinct from the nucleus, and which had not been described before; and he suggested the possibility of these bodies being accessory nuclei. Since their discovery by Kurloff they have been subjected to much research; and papers describing various observations concerning them have been published by Burnett (1904), Staubli (1905), Goldhorn (1905), Ledingham (1906), Howard (1907), Pappenheim (1908), Patella (1908), Hunter (1909), and Schilling (1911).

Kurloff noticed that when the blood containing these bodies was fixed and stained, they contained a nucleus-like structure staining with nuclear dyes, but he believed them to be vacuoles formed by a secretion product of the cells which held them. Ehrlich (1906) also thought that Kurloff's bodies represented some 'Secretstoff.' Dr. Ledingham, to whom I am indebted for much information, seems to have been the first to suggest the possibility of their parasitic nature, and he mooted an analogy to the *Cytorryctes variolae* or *vaccinae*. Goldhorn (1905) boldly called them leucocytozoa. The most recent work published on the subject is that of Schilling (1911). He has examined these bodies by 'vital' staining with Azur, and he has described some of the earlier stages of their development while in the mononuclear leucocytes

* Read before the Royal Society on February 29, 1912, and reprinted from the Proc. Roy. Soc., B, Vol. 85, pp. 67-72.

(lymphocytes). He believes that the rod stage precedes the granule stages, and this has caused him to adhere to the opinion that Kurloff's bodies must be classed with the Chlamydozoa, symbiotic structures, or vaccine inclusions.

Early in 1911, while examining a guinea-pig's blood by a new jelly method of examination of blood cells, H. C. Ross saw Kurloff's bodies, and pointed out to me that the method demonstrated the probability of their parasitic nature. The new method, which was devised partly at the suggestion of Sir Ronald Ross, K.C.B., has already been fully described (H. C. Ross, 1909); the bodies then seen were in the earlier stages of their development. But the inclusions stood out so clearly by this method that I determined to continue the observations, for this technique seemed to show details of structure which had not been described before; and since by the new process the bodies can be subjected to the action of various stains and chemical agents there was a possibility of the phases of their development being observed. I may state that I have now been able to convince myself that these bodies are living parasites of the mononuclear white corpuscles (lymphocytes), and henceforth in this paper I propose to call them such.

I use a modification of the original jelly method—it is as follows:—A 2 % solution of agar in water is boiled, sterilised and filtered. To 5 c.c. of the filtrate is added 0·5 c.c. of a 10 % solution of sodium chloride in water, and 0·5 c.c. of a 1 % solution of Azur II in water. The total bulk of the mixture is made up to 10 c.c. in a test tube. When molten, a small quantity of the jelly is allowed to spread itself in a thin film on a microscope slide and to cool and set. Then a drop of guinea-pig's blood (or citrated blood) containing Kurloff's bodies (about 90 per cent. of the guinea-pigs examined by me, and which were obtained from dealers in England, are infected) is placed upon a cover-glass, and this is inverted on to the set jelly. The blood spreads out between the cover-glass and the surface of the jelly, and, after an interval of five minutes, during which the blood corpuscles come to rest, the specimen may be examined under the higher powers of the microscope. After a further interval of a few minutes—the exact period varying slightly with the temperature of the room—the granules of the leucocytes begin to stain, after which their nuclei gradually stain a deep blue;

the contours of the erythrocytes, as well as those of the leucocytes, show up clearly, and the method is a pretty example of *in vitro* staining. In some of the larger mononuclear cells the colourless parasites will be noticed at one side of the protoplasm. These parasites are inside the cell, because the shape of the nucleus of the lymphocytes is moulded according to the size of the parasite, which grows larger as it develops—in its youngest stages it is small, while in its last intracellular stages it bulges the lymphocyte cell well and squeezes the nucleus into a small space; this point is of interest because, as Hunter has shown, Patella claimed that Kurloff's bodies lie upon and not in the lymphocytes. In cells containing the larger parasites smaller vacuoles can also be seen; these latter always remain clear and transparent even when examined on stain-containing jellies, and they vary in numbers, and slightly in size, in different examples. It has been suggested that these smaller, subsidiary vacuoles are polar bodies, but more probably they contain excretory products of the lymphocytozoa into the cytoplasm of their hosts, for they become larger and more numerous as the parasite grows.

When examined on the jelly, and immediately before the staining of the nucleus of the leucocytes, the contents of the parasites begin to stain*—the internal chromatin structure of the spherical sac embedded in the lymphocytes' cytoplasm becomes purple and remains stained for several hours, so that its examination is readily made. If the bloods of a number of infected guinea-pigs are watched in this manner from day to day what appear to be the successive stages of the growth of the parasite in the lymphocyte can be seen and drawn; but the leucocytes of a single animal at any particular moment contain, usually but not always, parasites in the same stage of development. The cycle, however, can be followed by observing the blood of one guinea-pig hourly.

---

*It must be emphasised that if the jelly contains excess of salts or impure stains, the wall of the parasite will stain in an irregular manner, and then patches of stain will hide its contents. Furthermore, if the blood on the jelly dries, or if the blood is fixed in any way, the same thing occurs. Similarly, patchy staining is obtained by the various fixed film methods in vogue, as, for example, Romanowsky's or Jenner's stains. Even Azur stain, when applied to the dried or fixed films of blood, will not demonstrate the details of the development of the parasite. No alkali should be added to the jelly.

The interpretation which I place upon the appearances I have seen are as follows:—The parasite presents itself, in the smallest phase of its intracorpuscular cycle, as a tiny translucent body embedded within the cytoplasm of the larger mononuclear blood corpuscles and near the periphery of those cells. Usually one of such bodies is present in any one cell, but occasionally two or even three parasites may occur in the same cell. The parasite, in this early stage, contains a double purple dot (Pl. VI, figs. 1, 2); in this phase it resembles the Leishman-Donovan bodies found in human leucocytes in cases of Kala Azar. When first seen the dot is motionless, but after a time on the jelly, as the lymphocyte host becomes disorganised, it may show some Brownian movement. In the next stage the parasite is larger, and the chromatin dot has divided into two or more dots until the sphere-like sac may be packed with them (fig. 3). Then each dot becomes dumb-bell shaped (fig. 4), and again, by a simple process of elongation, rod shaped (figs. 5, 6, 7). The parasite may contain one of these rods (fig. 9), or it may be full of them—the actual numbers varying in different examples. Sometimes a parasite may contain one or more rods, some dumb-bells, and some dots. But the size of the parasites increases steadily with these successive stages of the development of their contained chromatin (compare figs. 1 and 15). During the rod formation, the smaller subsidiary vacuoles already mentioned appear in the cytoplasm of the host cell (figs. 3, 5, 12); they never contain any chromatin and remain unstained. With its growth the parasite begins to compress the nucleus of the lymphocyte (figs. 13, 14), and the wall of the latter can be seen as a shell enclosing the parasite (figs. 14, 15, 16). The rods grow longer and thicker (figs. 8, 9, 10) until they stretch across the parasite, and their ends may be doubled against its wall, and they may then present in optical section an erroneous impression of flattening or a terminal bulging (figs. 8, 13, 14). In the next stage a stout flagellum grows out from both ends of the rod (figs. 8,11, 12, 13), which becomes rolled up in a coil within the sphere (figs. 13, 14, 15). The rod with its two flagella splits longitudinally in its whole length (figs. 8, 12), and this process of splitting takes place again and again. The fission throughout is always lengthwise, never transverse. A specimen in this stage will show the parasite, now equal in size to

the original dimensions of its host-cell, bulging the wall of the latter, compressing the nucleus into a small space, and containing within its interior a mass of woven, twisted, and intertwined purple threads, a conglomerate maze of worm-like spirilla stained red by the Azur dye (figs. 15, 16).

Arrived at its maturity, the parasite breaks away from the shell of its host-cell and then bursts, setting free the threads into the plasma (fig. 17). But the flagellate forms, owing to the fact that they are stained, are dead and motionless, and they may remain attached to the shrunken sphere sac, their ends waving in the currents set up.

It was found very difficult to demonstrate the motile, flagellate forms of the parasite when free in the blood. They cannot be seen then by the jelly method, because, probably, they stain momentarily as the trypanosomes do, and immediately die and become achromatic, and unless stained they are not visible. By the examination of ordinary wet films of the blood I was unable to demonstrate the presence of these free flagella, although a disturbance of the corpuscles was frequently seen. But the blood of some infected guinea-pigs, drawn under all aseptic precautions and examined by the dark ground illumination, showed free-swimming spirochaete-like bodies. It was not until the blood of highly infected guinea-pigs containing full matured lymphocytozoa was treated with an equal part of a 1 % solution of 'globin'* and incubated at 37° C. for eight hours that the free flagellate forms in the blood plasma could be fixed and stained by ordinary methods (fig. 18). Even by this process it is not always possible to demonstrate them, and the maceration involved gives them the appearance of spirilla with blunt ends. However, some of the spirilla obtained after the treatment with the 'globin' show the wavy outline of spirochaetes. Sir Ronald Ross was the first to suggest that these flagellate forms constitute the gametes of the parasite; this seems quite probable, though no separate female form has yet been noticed. It will be remembered that Lewis suggested that trypanosomes are sperms, and, perhaps, these spirochaete-like bodies are similar stages of a larger parasite.

*The filtrate of a solution of haemoglobin which has been precipitated by heat. H. C. Ross claims that this substance induces the division of certain cells.

What may possibly be the last phase of this parasite has occasionally been seen in preparations which had been submitted to the action of 'globin' for a further period of four hours. It is an object which resembles somewhat the trypanosome 'latent bodies' described by Moore and Breinl (fig. 19). Hunter has also mentioned the presence of amoeboid forms of this parasite being free in the plasma, but he does not picture them. These may be the form now drawn (fig. 19).

Dr. J. W. Cropper and I have repeated and can confirm the experiments of Ledingham (1906) and Hunter (1909), namely, that newly-born guinea-pigs do not show these lymphocytozoa in their blood. Although a pregnant animal may be markedly infected, the young, when born, possess no parasites. As has been already observed by these writers and by Schilling (1911), the number of parasites found in both the peripheral blood and in that of the internal organs of any one infected guinea-pig varies greatly from day to day. The parasites seem to appear in large numbers, to diminish, to disappear, and then, after a varying period of time, to reappear. Except for a slight anaemia, shown by the presence of an increased number of erythroblasts in the peripheral blood, the guinea-pigs do not suffer apparently. The livers of many of these infected animals show, however, single or multiple white patches of necrosis varying in size between that of a pin's head to that of a large pea, and extending into the substance of the organ. But we have no proof, as yet, of their direct relation to the parasite.

Fixed specimens of the various stages of the development of this parasite may be made by substituting an equal amount of a 1 % solution of caustic soda in the jelly for the sodium chloride solution. By this means the red blood corpuscles are laked, but the nuclei of the leucocytes and the chromatin of the lymphocytozoa stain well. The cover-glass can then be lifted from the jelly and mounted while still wet in Canada balsam. Many of the leucocytes with the contained parasites will adhere to the cover-glass and will retain their stain.

Since writing this paper, Hindle has published a preliminary note (Hindle, 1911), 'On the Life-cycle of *Spirochaeta gallinarum*.' He asserts that these spirochaetes possess an intracellular stage within the cells of the Malpighian tubes of the tick, *Argas persicus*.

In view of the life-history of this lymphocytozoon of guinea-pigs his work is of great interest.

I have to express my indebtedness to Dr. J. W. Cropper and to Dr. H. Bayon for their help in these researches; the latter was the first to recognise the free-swimming, spirochaete-like bodies. I also wish to thank Professor Minchin and Dr. Martin for much help and advice and the interest they have taken in this work.

SUMMARY

Kurloff's bodies are parasites, lymphocytozoa inhabiting only the mononuclear cells of the guinea-pig's blood.

These lymphocytozoa have an intracorpuscular stage, and ultimately give rise to free-swimming, spirochaete-like bodies, which may be gametes.

The development of the spirochaete-like body is demonstrated.

The name *Lymphocytozoon cobayae* is suggested for this parasite.

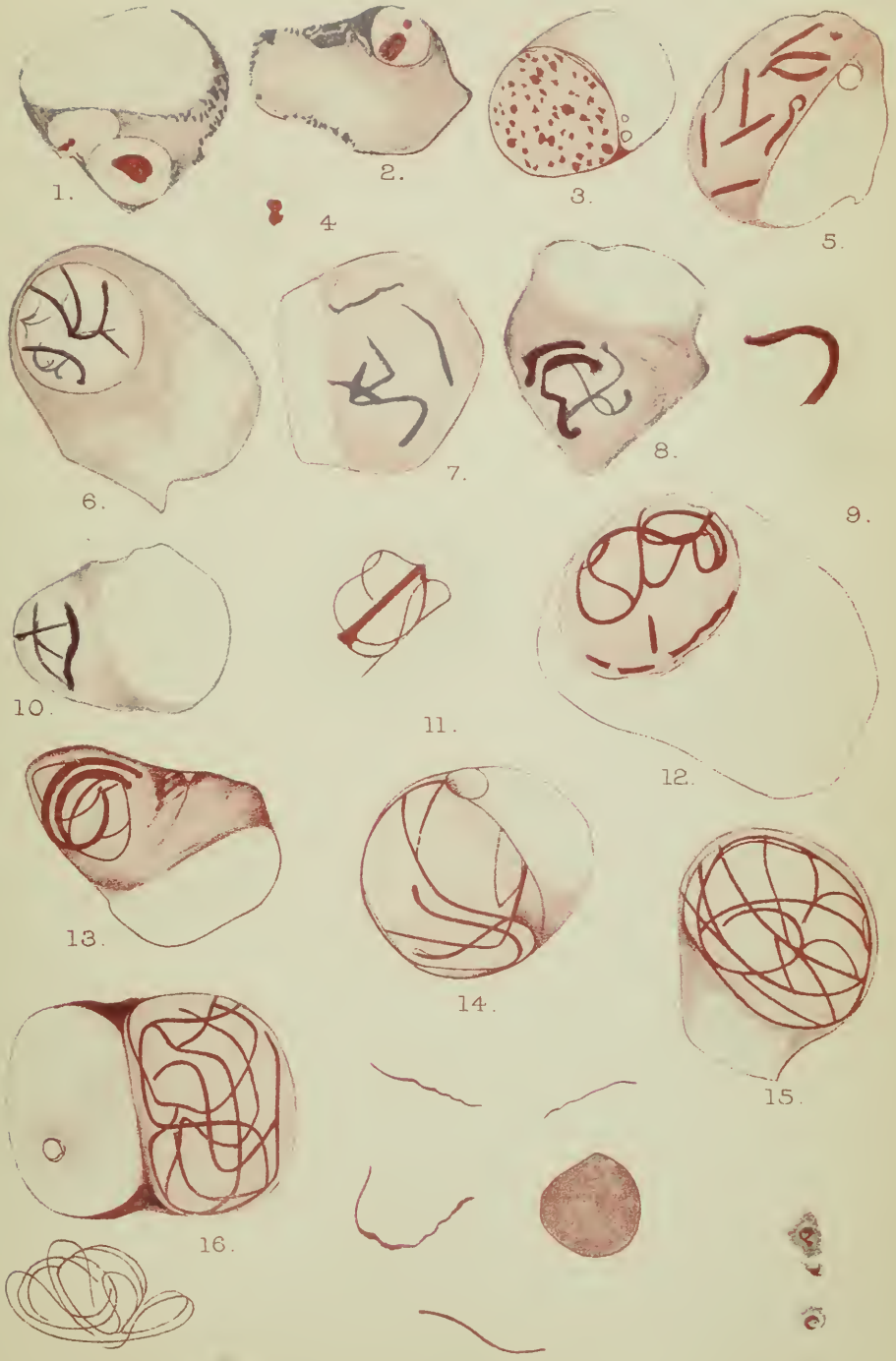
REFERENCES

Burnett (1904). Journ. Med. Research.
Ehrlich (1906). Quoted by Ledingham, Lancet, London, June 16.
Goldborn (1905). New York Path. Soc. Proc.
Hindle (1911). Parasitology, vol. 4, No. 4.
Howard (1907). Journ. Med. Research.
Hunter (1909). Univ. Penn. Med. Bull., Phila., vol. 22.
Kurloff (1891). Ehrlich's ' Die Anaemie.'
Ledingham (1906). Lancet, London, June 16.
Pappenheim (1908). ' Folia Haematologica.'
Patella (1908). Berliner klin. Woch.
Ross, H. C. (1909). Roy. Soc. Proc., B, vol. 81, p. 97.
Schilling (1911). Centralb. f. Bakt., orig., vol. 58, Part 4.
Staubli (1905). Deut. Archiv f. klin. Med.

PLATE VI

Lymphocytozoon cobayae.

For Explanation of Plate see text.



Miss E. Barry del. ad nat.

Huth Lith^r London

DEVELOPMENT OF A LEUCOCYTOZOON OF GUINEA-PIGS.

[From PARASITOLOGY, Vol. IV. No. 2, July 18, 1911]

AN IRON-HAEMATEIN STAIN.

WITH REMARKS ON THE GIEMSA STAIN.

By HARALD SEIDELIN, M.D.

(*From the Lister Institute, Zoological Department.*)

Plate V.

For general purposes, especially in haematological work, the Giemsa method, based on the Romanowsky principle, is certainly the most satisfactory stain for Protozoa. For certain structures, however, and in general as a control of the Giemsa stain, it is always necessary to have at one's disposal another method which will give particularly clear pictures of the nuclear elements (see *e.g.* Minchin, 1909). Of the different methods which are employed for this object the iron-haematoxylin stain is probably the most valuable and the most frequently used. There are, however, several circumstances which are unfavourable to its use as a universal stain. Its principal disadvantage is, that a differentiation is essential: the preparation is first deeply over-stained and the colour is afterwards extracted to the degree which is deemed convenient in each particular case. This extraction is always somewhat irregular, so that the observation, especially of intra-corpuscular parasites, is sometimes made very difficult, and it is not often possible to obtain in a single specimen a satisfactory stain of all the different elements. This difficulty is enhanced by the fact, that the red blood-corpuscles generally retain a deep grey, or almost black, colour. Further, a rather serious drawback is that in sections where greater masses of blood, and particularly blood-clots, are present, it is almost impossible to distinguish either intra- or extra-corpuscular parasites; to make such masses of blood

transparent it is necessary to differentiate so much, that the nuclei are also decolourized. A certain inconvenience is also experienced because of the long duration of the staining process, at least 30 hours when the original Heidenhain's method (1896) is adhered to; and although the more recent modifications, especially that of Rosenbusch (1908), have to a great extent overcome this difficulty, they still suffer from the two other disadvantages referred to.

I have therefore endeavoured to develop a quick and reliable stain of a similar nature, which does not require a differentiation. Beginning with the fact that haematein, as a rule, stains less diffusely than haematoxylin, I tried systematically mixtures of alcoholic haematein solutions and watery solutions of lithium carbonate, and obtained an excellent colouration of the tissue-nuclei, but did not find it satisfactory for Protozoa. The following formula gave the best results, staining 5 minutes:

Haematein sol. (1 % in 96 % alc.)	1 c.c.
Distilled water	4 c.c.
Saturated aqueous solution of lithium carbonate	5 drops.

Afterwards different combinations of iron-alum and haematein were tried, but always gave diffuse stains, so that a differentiation could not be dispensed with. Then I proceeded to experiment with alcoholic haematein and watery iron-perchloride solutions, after the manner of Weigert's iron-haematoxylin method (1904), which is known as an excellent nuclear stain for histological purposes, but as a rule does not stain Protozoa well. The following combinations were tried, A being a 1 per cent. solution of haematein and B the iron-perchloride solution of Weigert:

4 A + 1 B	diffuse stain
3 A + 2 B	good, both for sections and films
2½ A + 2½ B	good for sections, but too weak for films
2 A + 3 B	faint
1 A + 4 B	very faint.

I shall now describe the necessary details concerning the process. It is evidently only a slight modification of Weigert's method, but I believe that the difference will be found to make the present method useful in a certain number of cases, where Weigert's cannot be used.

Fixation. Sublimate-mixtures give the best results. I prefer Schaudinn's liquid: two parts of a saturated aqueous solution of mercury

bichloride and one of absolute alcohol. When this fixation is used, the pieces of tissue must be very thin, not more than 2–3 mm., but squares of about one cm. may be allowed. The tissues should be left in the fixative for 12 hours or more—two hours are sufficient for coverslip-films—but both may remain in it for over 24 hours without any bad effect. The fragments are then passed into 60 % alcohol, and subsequently into 70 % alcohol containing a small quantity of iodine, sufficient to give it a pale yellow colour; thereafter to 90 % alcohol and absolute alcohol, allowing one day in each of the four liquids. Clear thoroughly in xylol, pass to xylol-paraffin then paraffin, and finally embed quickly in pure paraffin. The sections should be cut as thin as is compatible with the particular purpose in view. For trypanosomes it is generally not advisable to use sections of less than five μ in thickness. They should be fixed on clean slides after simply floating them on warm distilled water (about 40° C.) and without using any fixative such as albumen glycerin. Films are treated in the same way, only they are not brought further forward than 90 % alcohol. I have also stained formalin-fixed sections with fairly good results. Osmic vapour fixation of films has so far not proved successful, but I have not had the opportunity of trying to bleach these preparations with hydrogen peroxide previously to the staining, which might well modify the results. On the other hand, the bleaching of sections of tissue fixed in Flemming's liquid has not made it possible to obtain a good stain.

The staining process. After dissolving the paraffin in xylol the sections are passed through alcohols of diminishing strength to tap water (or distilled water). This is important, as they do not stain well when taken directly from alcohol into the stain, in spite of the latter being an alcoholic solution. The staining liquid is prepared about 15 minutes before being used, as it does not stain well immediately after mixing; it may be kept for about two hours, but will not stain after a longer period. It is prepared by thoroughly mixing three parts of solution A and two of B. The solution A is prepared by adding one gram of pure haematein (Grübler) to 100 c.cs. of 96 % alcohol, and shaking repeatedly without heating. The whole quantity is not dissolved, but the solution is preserved with the residuum and, if not completely transparent, filtered before use. The solution B is composed as follows, after Weigert: perchloride of iron 4, hydrochloric acid 1, distilled water 100. The slides are left for about five minutes in the mixture, with the sections downwards, and for this purpose there

are in use in Prof. Minchin's laboratory some very convenient glass-dishes of the exact size of a slide, so that a comparatively small quantity of the staining solution is necessary. A longer time than five minutes is only very rarely necessary, but the staining may be controlled at any moment by examining the preparation with a low power after washing, and the slide may then, if necessary, be returned to the mixture. For showing the structure of certain nuclei, for instance of lymphocytes, an even shorter time in rare cases may be preferable. The sections are washed in tap water for several minutes; a longer washing, even of several hours, does not harm them, and may sometimes be useful in making the colour blacker, if it is too blue. They are subsequently taken through alcohols of increasing strengths to absolute and then xylol, and are mounted in Canada-balsam, or Damarlack.

Results. The stain has been tried on the different materials containing Protozoa, which have been available at the present time, especially on smears of rat-blood containing *Trypanosoma lewisi*, also on the contents of the digestive tube of leeches containing trypanosomes from fresh-water fishes; most of the latter forms were very small, and many of them extremely slender and very difficult to stain by any method. The stain was further applied to many sections, *e.g.* of leeches containing the above mentioned trypanosomes, and of human spleen infected with *Leishmania donovani*; also some sections without Protozoa were treated with the stain in order to try its value as an ordinary histological method. The tests, to which the methods have been subjected, have therefore been varied, if not very numerous, and several of them have been rather severe. As a rule it may be said that the stain has given the same results as the iron-haematoxylin method, but in much less time. In some cases it has proved distinctly superior, especially in some of the sections of leeches, which contained compact masses of half digested blood; an excellent result was obtained by the present method, whilst the preparations stained after Heidenhain or Rosenbusch were quite useless, as either the whole mass presented a nearly uniform black colour, or else, when the decolouration was carried far enough to make out details, the trypanosome nuclei were decolourized also.

The karyosome and chromatin-structures stain black, the kineto-nucleus being especially dark; the flagellum is grey and shows very sharply whilst the protoplasm takes a pale grey colour. As mentioned above, instead of the proportion of 3 to 2, equal parts of the two solutions may be used in sections when a particularly strong contrast

is desirable. The protoplasm then remains nearly unstained, the structure of the tissue-nuclei is shown beautifully, and it deserves especial mention, that the limits between the cells in the several different types of epithelia are remarkably well defined.

For histological purposes a combination of this stain with the acid fuchsin—picric acid—acetic acid solution of Hansen (1898) gives far clearer pictures than the so-called van Gieson's stain as ordinarily employed. It may of course also be combined with any other counter stain, just as the ordinary haematoxylin-method, but generally with better results, as the protoplasm comes out more sharply in the colour of the counter stain, because it absorbs haematein to a much less degree than it does haematoxylin.

I consequently believe, that the staining process will not only be a useful substitute for Heidenhain's method in protozoological work, but also that it may take the place of the different haematoxylin-combinations in most cases, especially as it is both rapid and easy to carry out. However, as to its principal advantages, I may emphasize the fact that it gives a good stain in certain cases where no other haematoxylin-method can be used, and that it does away with all differentiation, a circumstance which adds greatly to the reliability of any stain. The rapid execution will be of value especially for work in tropical countries, where it is always inconvenient to keep preparations for a long time in watery solutions.

This method certainly does not compete with the Romanowsky stains, especially the Giemsa and Leishman methods, which have a field of their own in the rapid diagnosis of malaria and other blood diseases, and which have the great advantage of not producing any confusion between chromatin and pigment, between which it is sometimes difficult to distinguish in sections stained by iron-haematein, or haematoxylin. In more detailed work the use of both the Giemsa and the iron-haematein-stains will generally be necessary.

In Pl. V some figures are given, which illustrate the results obtained. The method may be summarized as follows:

I. *Sections.*

1. Fixation in sublimate-alcohol with ordinary subsequent operations, embedding in paraffin.

2. Sections 5 μ in thickness, or less. Sections to be fixed on slides without any medium.

3. Passage through xylol, and alcohols of diminishing strengths to distilled, or tap water.

4. Staining for 5–10 minutes in a mixture of 3 pts. Solution A, and 2 pts. Solution B, which has been prepared not less than 15 minutes and not more than 2 hours. Sometimes equal parts of A and B may be used.

5. Washing in tap water for 5 minutes or more.

6. Passage through alcohols of increasing strengths to absolute and then xylol.

7. Canada-balsam or Damarlack.

II. *Films.*

The only differences are, that these are not brought up to xylol after hardening, but passed from 90 % alcohol to water, and that the mixture of 3 A + 2 B is the only one that gives good results.

After the staining they are treated exactly like sections, never being allowed to dry.

Remarks on the Giemsa stain.

The Romanowsky-stain has always been very difficult to apply to sections, in all its different modifications. Leishman's method gives fairly good results, but it is a great drawback that, for obtaining them, it is always necessary to be in possession of fresh serum (1904). Also Giemsa's method was originally intended only for smears which had been dried and fixed. But fortunately it now gives, when the principles laid down by Giemsa in his later publications (1910, 1 and 2) are strictly adhered to, excellent results also after wet fixation of films, and when applied to sections. In details some slight modifications may be made, whereby still better results may, in my experience, be obtained. As the method, which should certainly prove extremely useful, strangely enough does not seem to have come into general use, I shall describe the technique as I have now been using it on different materials for a considerable length of time. In fact, I had employed the acetone-differentiation a long time before the appearance of Giemsa's papers on that subject, but I only obtained the very best results after having become acquainted with the elaborate details, with which the author himself describes the method. Therefore the reader also must be

referred to the original publications, more especially to the first of the two referred to.

Fixation. Tissues must be fixed in sublimate-alcohol, as recommended above. No other method gives, as far as I have tried, useful results, and it must be especially mentioned, that the addition of acetic acid to the sublimate-alcohol detracts from the value of the stain. When films are used, a preliminary fixation may take place in osmic acid vapours, as recommended by Minchin (1909). The following procedure is the same as that described for the iron-haematein method. It is important not to use glycerin albumen for fixing the sections on the slides.

Staining. After taking the sections through the different alcohols I have found it preferable, instead of passing them first to distilled water, to leave them in tap water until transferred to the staining solution. Sometimes it is not necessary to use the somewhat complicated process of dissolving any traces of sublimate that may remain, which Giemsa recommends. If very thin fragments of tissue are fixed the sublimate is completely taken away by the iodine-alcohol which is employed during the hardening procedures; but if only few sections are at one's disposal it may be the wiser course not to omit this precaution. A prolonged staining gives the best results. I generally use a dilution of 1 to 20 (*i.e.*, 1 drop of Giemsa's solution to each c.c. of water) for 1 hour, and then change to a dilution of 1 to 40 (1 drop to each 2 c.c.'s), in which the sections are left for about 20 hours, but sometimes I have left them for 2 or 3 days in the weaker solution and still obtained very good results. The water employed for the dilution must be distilled and have been exactly neutralized, or slightly alkalinized, after titration, with a haematoxylin solution as indicator, as Giemsa describes it. (The water must turn a faint blue in the course of from one to five minutes after the addition of one to two drops of an alcoholic haematoxylin solution to ten c.c.'s, or potassium carbonate solution must be added till that point is reached.) The water, which I have been using, has generally needed the addition to each ten c.c.'s of about two drops of a cold saturated potassium carbonate solution, but the actual proportion undoubtedly varies very much, so that a frequent titration is inevitable. An omission of this step in the technique invariably spoils the results. The slides are preferably stained with the sections downwards, using for that purpose the glass dishes mentioned above. It is also important, that both the stronger and the weaker staining solutions should be prepared immediately before being used; when left

even for a short time they lose in staining power. From the staining solution the preparations are passed to tap water, not to distilled water, and then into pure acetone, or this liquid is continually dropped on the slide until the desired differentiation is obtained. When the preparations have been strongly over-stained the decolouration may take a long time, very often half an hour or more, and then one seems to get the best results. The progress of the differentiation should be frequently controlled under the microscope. From pure acetone, which I prefer to the first mixture of Giemsa of 95 acetone and 5 xylol, the section passes into the second mixture of 70 acetone and 30 xylol, and after 5 minutes into a third mixture of 30 acetone and 70 xylol. It is decidedly better to use these three steps, instead of only two as Giemsa recommended. It sometimes happens, however, that the last named mixture of acetone and xylol becomes cloudy, but it seems to keep clear when both the xylol and the acetone are very pure; if it becomes cloudy at least equal parts of the two substances may be used. It is certainly not convenient to pass the specimens directly from 70 acetone and 30 xylol into pure xylol. After five minutes in each of the two mixtures pass into pure xylol, which should be changed once so that no trace of acetone may be left. The sections may be mounted in Canada-balsam, but I have for some time been using Damarlack, in which they should keep much longer without fading, if we can draw any conclusions from experience with other stains, which would seem to last much longer in Damarlack, as I have learned from Prof. C. J. Salomonsen in Copenhagen.

Results. This method has been tried during the last few months on the same material as the iron-haematein stain and at an earlier date on a good deal more. The different elements stain in the same way as by the ordinary Giemsa method and the results have been uniformly good on material which had been fixed in sublimate-alcohol, whilst after formalin and osmic acid they have been as constantly negative. If there is any difference, the chromatin stains still better than in dry preparations, and I can quite confirm Giemsa's assertion, that in blood smears and other films better results can be obtained after wet fixation than by the dry method.

It may be convenient to give a brief summary of this technique also, though the differences from the one given in Giemsa's paper are very small:

1. Fixation, hardening, and embedding as above.
2. Sections of a uniform thickness of 5 μ, or less.
3. Xylol, alcohols of diminishing strengths, tap water.

4. Water, to which Lugol's iodine-solution has been added in the proportion of 2 to 100, 5–10 minutes.
5. Water.
6. 0·5 % solution of sodium thiosulphate, 5–10 minutes.
7. Tap water, 5 minutes or more.
8. Diluted Giemsa-solution 1 : 20 in distilled water, which has been neutralised (haematoxylin-indicator) with potassium carbonate solution, 1 hour.
9. The same 1 : 40, 20–24 hours.
10. Wash in tap water.
11. Differentiate in pure acetone. (Control under microscope.)
12. Acetone 70, xylol 30, 5 minutes.
13. Acetone 30, xylol 70, 5 minutes.
14. xylol, 5 minutes.
15. Fresh xylol.
16. Damarlack.

I wish to express my thanks to Prof. E. A. Minchin for his kind permission to work in his laboratory, and to Dr H. M. Woodcock and Miss Muriel Robertson for material and for having tried the stain. I am also indebted to Dr J. W. W. Stephens for some material.

REFERENCES.

Giemsa, G. (1910). Ueber die Färbung von Schnitten mittelst Azur-Eosin. *Deutsche med. Wochenschr.* No. 12, p. 550.

—— Zur Färbung von Feuchtpräparaten und Schnitten mit der Azureosin-methode. *Centralbl. f. Bakteriol. u. Parasitenk.* Abt. 1, Orig. Vol. liv. p. 489.

Hansen, Fr. C. C. (1898). Eine zuverlässige Bindegewebsfärbung. *Anat. Anz.* Vol. xv. p. 151.

Heidenhain, M. (1896). Noch einmal über die Darstellung der Centralkörper etc. *Zeitschr. f. wissenschaftl. Mikr.* Vol. xiii. p. 186.

Leishman, W. B. (1904). A method of producing chromatin-staining in sections. *Journ. of Hygiene*, Vol. iv. p. 434.

Minchin, E. A. (1909). The structure of *Trypanosoma lewisi* in relation to Microscopical Technique. *Quart. Journ. Micr. Sci.* Vol. liii. p. 755.

Rosenbusch, F. (1908). [Verhandl. d. deutschen tropenmedizinischen Gesellschaft.] Beihefte z. *Arch. f. Schiffs- u. Tropen-Hygiene*, Vol. xii. p. 277.

Weigert, K. (1907). Eine kleine Verbesserung der Haematoxylin—van Gieson—Methode. *Zeitschr. f. wissenschaftl. Mik.* Vol. xxi. p. 1.

EXPLANATION OF PLATE V.

All the figures have been drawn from iron-haematein preparations with Zeiss's prism, using Zeiss's apochr. obj. 3 mm. or 2 mm. and compens. oc. 18. Figs. 1–5 × 2000, Fig. 6 × 3000. My thanks are due to Miss M. Rhodes, of the Lister Institute, for executing the drawings.

Fig. 1. *Trypanosoma lewisi* in film, using the wet method. (Fixation in Schaudinn's liquid.)

Figs. 2, 3. Fish trypanosomes from digestive tube of leech (*Hemiclepsis marginata*); wet method. (Schaudinn's liquid.)

Fig. 4. The same in the interior of the half digested mass of blood, in section of leech. (Schaudinn's liquid, paraffin section.)

Fig. 5. Part of digestive tube of the same leech. To the right are the epithelial cells; to the left the partially digested blood, and between both the trypanosomes, of which only a part of the body is to be seen, most of them having been cut through in an irregular manner.

Fig. 6. *Leishmania donovani* in section of spleen. The outline of the nucleus of the host cell has come out too darkly in the reproduction. (Formalin, paraffin.)

Illustrating the paper by Dr H. Seidelin.
(Description: see p. 103.)

[*Reprinted from the Journal of Physiology.*
Vol. XLIV. No. 3, *May* 6, 1912.]

THE ACTION OF THE LIVER ON THE SIMPLER SUGARS.

By IDA SMEDLEY, *Beit Memorial Research Fellow.*

(*From the Lister Institute.*)

In spite of the investigations of numerous workers, the mechanism by which dextrose is transformed in the body remains completely unknown. No certain evidence has yet been obtained that sugar can be changed by the action of any isolated organ, if the experiments carried out on the isolated heart, in which alone the disappearance of sugar has been satisfactorily established, are excepted (Locke and Rosenheim[1]). The action of the body organs on the simpler sugars has however been little investigated. Experiments with the simplest sugar, glycollic aldehyde, were carried out by P. Meyer[2] who injected subcutaneously doses of from five to ten grams; neither glycollic aldehyde, glycollic acid nor glyoxylic acid was identified in the urine but when doses of more than five grams were given, dextrose was detected. Injection of the corresponding glycol ($CH_2OH—CH_2OH$) led to the excretion of glycollic acid in the urine.

I have carried out experiments in which solutions of glycollic aldehyde ($CH_2OH.CHO$), glyceric aldehyde ($CH_2OH.CHOH.CHO$), di-hydroxy-acetone ($CH_2OH.CO.CH_2OH$), xylose and dextrose were subjected to the action of minced liver tissue.

The glycollic aldehyde used was prepared by the method described by Fenton[3]. The solution obtained by heating a solution of dioxymaleic acid was used without isolating pure glycollic aldehyde.

The solution of glyceric aldehyde was obtained by Wohl's method[4]; the aldehyde was not isolated in the pure state but the syrup obtained on concentrating the solution of the aldehyde under diminished pressure was at once used. In some of the experiments, the melting-point of the osazone showed that partial conversion into acrose had occurred; in these, the reducing power of the solution was not entirely removed after incubating with the liver tissue. When however the

[1] Locke and Rosenheim. This *Journal*, xxxvi. p. 205. 1907.

[2] *Ztschr. physiol. Chem.* xxxviii. p. 134. 1903.

[3] *Journ. Chem. Soc.* lxvii. p. 774. 1895.

[4] Wohl. *Ber.* xxxi. pp. 1796, 2394. 1891; Wohl and Neuberg, xxxiii. p. 3095. 1900.

melting-point of the osazone corresponded with that of the pure glycerosazone (132°), the reducing power of the sugar solution completely disappeared after incubation.

The method adopted was as follows: 40 c.c. of a solution of the sugar to be investigated and 10 c.c. of a normal solution of sodium-bi-carbonate were added to 50 grams of minced pig's liver and the mixture saturated with CO_2. After shaking for from 15 to 30 minutes, the mixture was left from three to four hours at 37°. Ten c.c. of normal sulphuric acid and 50 c.c. of dialysed iron solution were then added and the whole made up to a known volume, generally to between three and five hundred c.c. The reducing power of the filtrate was determined by Bertrand's method. Control experiments were carried out in which an equal weight of liver and an equal volume of the sugar solution separately underwent similar treatment, and the reducing power of the three filtrates at the same degree of dilution was compared.

In the following table the number of milligrams of copper reduced by 20 c.c. of the sugar solution, are shown.

Substance		Control sugar	Control liver	Control sugar and liver	Sugar and liver after incubating 4 hrs. at 37°	Sugar removed
Dextrose	(1)	122 mg. Cu	14 mg.	136 mg.	137 mg.	+1 mg.
$C_6H_{12}O_6$		122	12·5	134·5	136	+1·5
Acrose	(1)	65	20	85	83	−2
$C_6H_{12}O_6$		66	20	86	84	−2
Xylose	(1)	—	—	102	96	−6
$C_5H_{10}O_5$						
Glyceric Aldehyde	(1)	50	125	175	148·5	−26·5
CH_2OH		48	125	173	146·5	−26·5
|	(2)	53	25	88	46	−42
CHOH	(3)	35	34	69	37	−32
|						
CHO						
Dioxyacetone		75	80	155	158	+3
CH_2OH			79	154	151	−3
|				54	51	−3
CO						
|						
CH_2OH						
Glycollic Aldehyde	(1)	50·5	10	60·5	36	−24·5
CH_2OH		51·5	11	62·5	37	−24·5
|	(2)	48·5	3·5	52	17·5	−35·5
CHO		48·5	5	48·5	17	−31·5

The solution of glyceric aldehyde in (3) gave an osazone, m.pt. 131–2, and had therefore not undergone polymerisation to a hexose. In (1) and (2) partial transformation to acrose had taken place, an osazone, melting between 150 and 160, being obtained.

It will be seen that no change in the reducing power of the solutions of xylose and dextrose was observed, nor did the solutions of di-hydroxy-acetone show any alteration in reducing power, but the reducing power of the solutions of glycollic and glyceric aldehydes disappeared or was markedly diminished. After treating a solution of glyceric aldehyde with minced liver for from three to four hours, the aldehyde completely disappeared; the solution no longer reduced at ordinary temperature nor did it give the characteristic phloroglucinol reaction. The action of the liver is not comparable with that of dilute alkalis on glyceric aldehyde, for whereas the latter readily effect the transformation of this aldehyde into acrose, the substance formed by the action of the liver is without reducing properties and no longer therefore contains an aldehyde or ketone group.

An enzyme capable of transforming glyceric aldehyde but incapable of acting upon dextrose must therefore be considered to be present in the liver. It is possible that this reaction may be considered to be a step in the normal metabolism of sugar and that the rapid removal of glyceric aldehyde by the liver may have prevented its detection. No evidence which would indicate the transformation of sugar to glyceric aldehyde in the body has however yet been obtained.

It has been shown by Parnas that an enzyme, termed by him aldehyde mutase, is present in the liver capable of converting fatty aldehydes quantitatively into the corresponding alcohol and acid[1]

$$2RCHO + H_2O = RCH_2OH + RCOOH.$$

Although isovaleric aldehyde and the higher fatty aldehydes are quantitatively transformed into the corresponding alcohols and acids by the liver tissue *in vitro*, yet the comparatively stable higher fatty alcohols have been detected neither in the liver nor in the blood. It is still therefore a matter of uncertainty whether fatty aldehydes are normally formed in the body and there undergo the Canizarro reaction as above described.

Since glycerol is the alcohol normally occurring in the body, it is possible that the work of the aldehyde mutase may be to produce glycerol and glyceric acid from glyceric aldehyde and that this may occur as a normal stage in carbohydrate metabolism. I am at present investigating whether the action of the liver on the simpler sugars is similar to that described by Parnas on fatty aldehydes.

[1] *Biochem. Ztschr.* XXVIII. p. 274. 1910.

[*Reprinted from the* Proceedings of the Royal Society, B. Vol. 84]

The Chemical Action of Bacillus cloacæ (*Jordan*) *on Glucose and Mannitol.*

By James Thompson.

(Communicated by Arthur Harden, F.R.S. Received November 22, 1911,—Read February 1, 1912.)

(From the Biochemical Department, Lister Institute.)

The close relationship of *B. cloacæ* (Jordan) to *B. lactis aërogenes* (Escherich) suggested the investigation of the chemical action of the former on glucose and mannitol. The two organisms are lactose-fermenting bacilli, allied to *B. coli communis*, and showing a close resemblance to each other in their biological characteristics. *B. lactis aërogenes* is a non-motile, Gram-negative, non-liquefying bacillus, a facultative anaërobe which produces acid and clotting in milk. *B. cloacæ* is a facultative anaërobic bacillus, actively motile, Gram-negative, slowly liquefying gelatine, and producing acid and clot in milk. The chief biological characters of the organisms will be clearly seen in the following table, in which + means acid and gas, — no action :—

	Glucose.	Lactose.	Cane sugar.	Dulcitol.	Dextrin.	Inulin.
B. cloacæ	+	+	+	—	+	—
B. lactis aërogenes ..	+	+	+	—	+	—

Harden and Walpole* have already fully investigated the products of the decomposition of glucose and mannitol by *B. lactis aërogenes*, and a comparison of their results with those to be obtained from *B. cloacæ* presented a problem of considerable interest, owing to the fact that both organisms give the Voges and Proskauer reaction. This reaction is due to the presence of acetylmethylcarbinol, which is closely related to butylene glycol, a substance which had been found as one of the products of the fermentation of glucose by *B. lactis aërogenes*. The organism was grown anaërobically in a medium containing 1 per cent. of Witte peptone and 2 per cent. of the sugar in the presence of chalk. The products were examined by the method outlined by Harden† in his investigation of the action of *B. coli communis* on glucose.

An alteration in the method of collecting the evolved gases was made, with the object of eliminating the error involved in collecting over saturated brine, in which carbon dioxide is slightly soluble. The collecting apparatus

* 'Roy. Soc. Proc.,' 1906, B, vol. 77, p. 399, and 1911, B, vol. 83, p. 272.

† 'Chem. Soc. Trans.,' 1901, p. 610.

used consists essentially of an evacuated flask of about 5 litres capacity, and has been fully described in a previous paper.*

A. *Action of* B. cloacæ *on Glucose.*

The substances produced by the action of *B. cloacæ* on glucose were found to be the same as those found in the case of *B. lactis aërogenes*, viz., acetic acid, lactic acid, succinic acid, formic acid, ethyl alcohol, carbon dioxide, and hydrogen. No trace of marsh gas was detected. As in the case of *B. lactis aërogenes* the culture medium at the end of the fermentation gave Voges and Proskauer's reaction, and there was also production of butylene glycol, amounting to 19 per cent. of the sugar. The relative proportions of the products of fermentation differed considerably from those of *B. coli communis* and, in a less degree, from those of *B. lactis aërogenes*. These variations are shown in the following tables. The actual percentages by weight of the products on the glucose fermented in two separate determinations are given in Table I, Columns 1 and 2. For comparison, in Column 3 are given the results of a typical fermentation of glucose by *B. lactis aërogenes*, and in Column 4 those of a similar fermentation by *B. coli communis*. Table II shows the number of carbon atoms per molecule of glucose represented by each product.

On comparing the results given in Table I it will be seen that the ratio of hydrogen to carbon dioxide by volume, viz. 0·3 : 1, in the gas evolved from glucose by *B. cloacæ* is somewhat smaller than in the case of *B. lactis aërogenes* (= 0·42 : 1), and markedly less than for *B. coli communis* (= 1·19 : 1). Theobald Smith† gives the characteristic ratio for *B. lactis aërogenes*

Glucose.

Table I.—Percentages.

	B. cloacæ.		*B. lactis aërogenes.*	*B. coli.*
	1.	2.	3.	4.
Alcohol	16·55	13·15	17·1	12·85
Acetic acid	1·17	3·04	5·1	18·84
Lactic acid	2·04	10·99	5·5	31·90
Succinic acid	2·03	1·74	2·4	5·20
Formic acid	4·31	3·20	1·0	0·0
Carbon dioxide	41·75	41·11	38·0	18·09
Carbon dioxide, c.c. per grm.	211·3	208·0	198·3	91·8
Hydrogen, c.c. per grm.	64·0	55·7	82·4	110·0
Ratio H_2/CO_2	0·3	0·27	0·42	1·19

* Harden, Thompson, and Young, 'Biochem. Journ.,' 1910, vol. 5, p. 230.

† Theobald Smith, 'Centralb. f. Bacteriol.,' 1895, vol. 18, pp. 1, 494, 589.

Glucose.

Table II.—Carbon Atoms.

	B. cloacæ.		*B. lactis aërogenes.*	*B. coli.*
	1.	2.	3.	4.
Alcohol	1·30	1·03	1·34	1·01
Acetic acid	0·07	0·18	0·31	1·13
Lactic acid	0·12	0·66	0·33	1·91
Succinic acid	0·12	0·11	0·15	0·32
Formic acid	0·17	0·13	0·04	0·00
Carbon dioxide	1·71	1·68	1·60	0·74
Total	3·49	3·79	3·77	5·11
Hydrogen, atoms per molecule	1·04	0·90	1·33	1·77

$H_2/CO_2 = 1:1$, but this result does not represent the actual ratio of the gases produced, owing to the solubility of the carbon dioxide in the liquid medium contained in the ordinary fermentation tubes which he employed. This source of error has been obviated, as already pointed out, by collecting the gases over mercury in an evacuated flask. Formic acid was found in the products obtained from *B. cloacæ* in far greater amount than in those from *B. lactis aërogenes*, while those given by *B. coli* are usually almost free from this substance. It is, however, probable that at least a portion of the carbon dioxide is derived from the decomposition of formate primarily formed as an intermediate product. A very marked difference in the relative proportions of alcohol and acetic acid produced by the three organisms will be noticed. While the molecular ratio alcohol/acetic acid for *B. coli communis** is 1, and for *B. lactis aërogenes* (average of three determinations) = 4, that for *B. cloacæ* was found in two experiments to be 18 and 6. The large difference between these results is due to the fact that only a very small amount of acetic acid is produced, and a small absolute difference in this produces a large change in the ratio. Succinic acid is produced by *B. cloacæ* in rather smaller amount than by *B. lactis aërogenes*, and in less than half the quantity given by *B. coli communis*. The amount of alcohol is approximately equal to that given by *B. lactis aërogenes*. A considerable deficiency of carbon in the fermentation of glucose by *B. cloacæ* was found, and, remembering the very similar biological characters of *B. cloacæ* and *B. lactis aërogenes*, butylene glycol was sought for.

* Harden, 'Journ. Hygiene,' 1905, vol. 5, p. 488.

Production of 2.3-*Butylene Glycol by* B. cloacæ.

A medium containing 50 grm. of glucose in 1 litre of a 1 per cent. solution of Witte's peptone, to which had been added 10 grm. of chalk, was inoculated with the bacillus. After six weeks' incubation at 37°, the liquid was distilled to dryness under reduced pressure at 40°. The dry residue was extracted with boiling absolute alcohol, and the alcoholic solution distilled at 40° under reduced pressure. The residue, weighing 10·6 grm., was fractionated at normal pressure. A fraction distilling between 178° and 184°, weighing 9·5 grm., which solidified completely in a freezing mixture, was obtained. That this substance was 2.3-butylene glycol was proved by converting a portion of it into diacetyl* by the action of bromine water under the influence of light. From 4·5 grm. butylene glycol was obtained 1 grm. diacetyl, from which was prepared the phenylosazone.† After recrystallisation from alcohol and water the latter was found to have a melting point 243°.

B. *Action of* B. cloacæ *on Mannitol.*

Considerable differences, while on the whole not so marked as in the case of glucose, are also found on comparing the results of the fermentation of mannitol by *B. cloacæ* with those of *B. coli communis* and *B. lactis aërogenes*. In Table III, Columns 1 and 2, are given the results of two separate determinations of the products resulting from the action of *B. cloacæ* on mannitol, and in Columns 3 and 4 are given for comparison the figures obtained by typical fermentations of mannitol by *B. lactis*

Mannitol.

Table III.—Percentages.

	B. cloacæ.		*B. lactis aërogenes.*	*B. coli.*
	1.	2.	3.	4.
Alcohol	27·45	26·48	32·5	28·1
Acetic acid	4·23	3·67	2·5	9·5
Lactic acid	2·64	2·24	8·6	18·6
Succinic acid	2·29	4·24	3·2	8·9
Formic acid	5·56	4·56	1·5	3·0
Carbon dioxide	29·02	31·20	35·5	28·44
Carbon dioxide, c.c. per grm.	146·8	157·8	180·3	143·0
Hydrogen, c.c. per grm.	110·2	116·9	138·3	167·0
Ratio H_2/CO_2	0·75	0·74	0·77	1·18

* v. Pechmann, 'Ber.,' 1890, vol. 23, p. 2427.

† v. Pechmann, 'Ber.,' 1888, vol. 21, p. 2754.

Mannitol.

Table IV.—Carbon Atoms per Molecule of Mannitol.

	B. cloacæ.		*B. lactis aërogenes.*	*B. coli.*
	1.	2.	3.	4.
Alcohol	2·17	2·10	2·57	2·22
Acetic acid	0·26	0·22	0·15	0·58
Lactic acid	0·16	0·14	0·52	1·13
Succinic acid	0·14	0·26	0·20	0·55
Formic acid	0·22	0·18	0·06	0·12
Carbon dioxide	1·20	1·29	1·47	1·16
Total	4·15	4·19	4·97	5·76
Hydrogen, atoms per molecule	1·80	1·91	2·26	2·7

aërogenes and *B. coli communis* respectively. Table IV shows the number of carbon atoms per molecule of mannitol represented by the various products. As in the case of glucose, a considerable deficiency in the carbon will be noticed; this is to be accounted for by the production of acetylmethylcarbinol and butylene glycol, a qualitative experiment having shown the presence of both these substances.

In the case of mannitol there is practically no difference in the ratios H_2/CO_2 for the two organisms *B. cloacæ* and *B. lactis aërogenes*. As with glucose, the amount of formic acid obtained from *B. cloacæ* is considerably greater than from either *B. lactis aërogenes* or *B. coli communis*. On the other hand, the opposite is to be observed in the figures given for succinic acid. A comparison of Tables I and III shows that *B. cloacæ* produces twice as much alcohol from mannitol as from glucose. This further confirms the suggestion previously made by Harden* that the formation of alcohol in these reactions is related to the presence of the terminal $CH_2(OH)\cdot CH(OH)$ group, which occurs twice in the molecule of mannitol, and only once in that of glucose.

* Harden, 'Chem. Soc. Trans.,' 1901, p. 610.

Harrison and Sons, Printers in Ordinary to His Majesty, St. Martin's Lane.

[From PARASITOLOGY, Vol. IV. No. 2, July 18, 1911]

A REPLY TO MISS PORTER'S NOTE ENTITLED "SOME REMARKS ON THE GENERA *CRITHIDIA*, *HERPETOMONAS* AND *TRYPANOSOMA*."

By H. M. WOODCOCK, D.Sc.,
Lister Institute, London.

In a recent number of *Parasitology*[1], Miss Porter, under the above heading, complains because, in the *Zoological Record* for 1909 (*Protozoa* division, p. 60), I put the generic names *Crithidia* and *Herpetomonas* in inverted commas, when applied to certain species; on this ground she charges me with allowing my personal opinions to influence me in my compilation of the *Record*, my opinions being, according to her, "extreme and decidedly opposed to the opinions of those who have worked personally on the parasites in question." Incidentally, Miss Porter makes it appear that my opinions *are* extreme and of little or no value.

I should not have troubled to reply to Miss Porter's remarks if she had not criticised my conduct of the Protozoa division of the *Record*. But as this criticism is calculated to mislead any general Readers who may be interested in the *Zoological Record* but are not particularly conversant with the point at issue, I propose to justify myself to them. I am sorry that in doing so I shall have to show that Miss Porter's method of criticism is not straightforward, but, on the contrary, distinctly oblique.

To begin with, I will refute the charge that my opinions are extreme. In the first place, Miss Porter quotes *part* only of the references to *Crithidia* and *Herpetomonas* given on p. 60 of vol. 46 of the *Record*. Immediately before the species she quotes, under the two respective headings, are the following references: "*Crithidia gerridis*, general account, Porter," etc.; and "*H.* (i.e. *Herpetomonas*) *vespae* sp.n., *H. jaculum*, Porter," etc. That is to say, the generic names are *not* put in

[1] Vol. iv. pt. 1, 1911, p. 22.

inverted commas when the references are to accounts of such parasites from non-blood-sucking Insects—including, it will be seen, the reference to Miss Porter's own paper. In these cases they are given as indicating real genera. It is only where such forms are described from blood-sucking hosts that the inverted commas are used. Again, to leave the *Record*, in my own article on Avian Haemoprotozoa, to which Miss Porter also refers, on p. 713 is the following sentence: "One or two cases have been described, however, of the occurrence of crithidial forms in what are alleged to be non-sanguivorous Insects (*e.g. C. gerridis*, from *Gerris fossarum*, Patton); such parasites may apparently be regarded as true Crithidiae, by which we may understand Flagellates that have developed a trypanomonad condition but which are restricted to an Invertebrate host." This sentence is on the page on which I mention my discovery of a Trypanosome in the blood of the sheep. Further, I quote the following sentences (p. 244) from my article, written about the beginning of 1908, on the Haemoflagellates in Lankester's *Treatise on Zoology*, vol. I. Protozoa, to which Miss Porter also makes reference: "Hence, summing up, there can be little doubt that certain of these parasites of mosquitoes, especially those with trypaniform characters, are connected with some Vertebrate host, just as are those of other blood-sucking Invertebrates. At the same time, it is also probable that some of the (typical) Herpetomonads found (*e.g.* those occurring in larvae, such as Patton's form, also certain forms described by the Sergents) are simply and primarily parasites of the Insect. Lastly, it is, of course, possible that such a parasite may have developed a trypaniform condition as an adaptation to the food of a sanguivorous Insect, without, however, having become able to live in the Vertebrate host; but so far no example of such a case is definitely known." (I may say in passing, with regard to the last sentence, that I would not go so far in that direction to-day; as I discussed in my recent paper on Avian parasites, I consider the occurrence of a trypaniform phase in a blood-sucking Insect is almost conclusive indication that the parasite in question is a Trypanosome.)

It is extremely doubtful, indeed, whether even any of the trypanomonad ("crithidial") forms found in blood-sucking Insects can be regarded as independent. It is true that I have not worked personally on genuine crithidial or herpetomonad parasites from non-blood-sucking Insects. But I have paid considerable attention to the characteristic developmental phases of blood-Trypanosomes, which occur both in cultures and in the Invertebrate hosts. And knowing what I do about such forms, as soon as I observed and studied "*Crithidia*" *melophagia* occurring

in several sheep-keds taken from the same sheep, the conclusion was irresistibly suggested to my mind that this parasite could be nothing else than the trypanomonad form of a Trypanosome in the sheep. At Miss Robertson's suggestion, she and I instituted a searching examination of the blood of that particular sheep, with the result that I found the Trypanosome whose presence was suspected.

Having regard to all the known facts, the only reasonable and logical view to take is that these parasites in blood-sucking Insects are phases of some Trypanosome, *especially where the Insects feed on animals known to harbour a Trypanosome* (*e.g.* rat-fleas, mole-fleas, sheep-keds, etc.). This is the view of commonsense. So far as proof goes, this view has already been conclusively proved by the work of Minchin and Thomson, in the case of the parasites of the rat-flea; and sooner or later it will be proved in other cases. Most assuredly, however, the *onus probandi* lies rather on those who hold the opposite view; and, so far as I am aware, neither Miss Porter nor anyone else has attempted to prove that any of these "*Crithidiae*" in blood-sucking Insects are true independent forms.

Secondly, the above view represents not only *my* personal opinion. It is also the opinion of the great majority of Protozoological workers, of whom I need only refer to Prof. Minchin, whose name is generally recognized as that of one of the foremost authorities on Trypanosomes and allied parasites. Miss Porter mentions Swingle as being opposed to this view. Swingle is certainly not now opposed to it; he is at any rate quite open-minded, and indeed if anything *in favour of* my view. In a recent paper on the transmission of *T. lewisi* by rat-fleas[1], etc., he says on p. 131, with reference to Strickland's account of *Crithidia ctenophthalmi*: "It should be noted that these fleas all came from rats infected with Trypanosomes, which fact strongly suggests that his Crithidiae were really transformed Trypanosomes." Again, in a footnote to p. 141, with reference to my discovery of the Trypanosome in the sheep, he says: "While there is considerable evidence favouring his conclusion, it seems to me there is still a possibility that *C. melophagia* is a true Insect Flagellate, which has never been successfully introduced into the sheep's blood." "Considerable evidence" in favour, it will be noted, and only a "possibility" against. Lastly, I am sure Dr Swingle will not object to my quoting a letter which I received lately from him: "I received your excellent article on Avian Haemoprotozoa soon after I had sent my work to the printer. It is interesting

[1] *Journal Inf. Diseases*, VIII. 1911, p. 125.

that you found a Trypanosome in the blood of the sheep. Since the Trypanosomes change over into Crithidiae in the digestive tract of Insects, it would not be surprising if experiment should reveal the fact that *C. melophagia* is got from the blood of the sheep."

From what I have shown above, therefore, two things will be clear to those who are really interested in the *Zoological Record.* In the first place, there is no truth in the assertion that my opinions on the subject are extreme; on the contrary, they agree with the view which most workers on it hold. Secondly, I am correct in having used and in continuing to use the terms *Crithidia* and *Herpetomonas* in inverted commas, when applied to parasites from blood-sucking Insects, signifying thereby that, according to our present knowledge, such forms are in all probability *not* to be regarded as belonging to truly independent genera, but are merely developmental phases of some Trypanosome. At any rate, I have received the assurance of the Secretary of the Zoological Society and of others on the matter. A Recorder is allowed the use of his own judgment in the case of debated points; and I can truthfully say that I endeavour to keep mine unbiassed and to compile the Protozoa division of the *Record* in accordance with the general tendency of opinion.

In conclusion I have only to say that the above justification is not intended as a personal reply to Miss Porter. As between Protozoologists, the only comment I should make upon her note would be in terms very similar to those in which I referred to Capt. Patton's "criticism" of my article on the Haemoflagellates, in my paper on Avian Haemoprotozoa, p. 715.

(Sonderabdruck aus dem »Zoologischen Anzeiger« Bd. XXXVIII. Nr. 22/23
vom 21. November 1911.)

On an unusual condition observed in Halteridium.

By H. M. Woodcock, D. Sc., Lister Institute of Preventive Medicine, London.

(With 22 figures.)

In a chaffinch which was well infected with *Halteridium* (*Haemoproteus*) *fringillae*, I have recently observed an interesting and peculiar condition of certain parasites. As this is the first occasion on which I have seen exactly this condition, notwithstanding a considerable amount of time and attention directed to the study of *Halteridium*, and as I am not aware of its having been noted by any one previously, I think it is worth a brief description.

In the first permanent preparations made[1] from the blood of this particular bird, many of the Halteridia, intermediate-sized forms as well as large ones, were found to have two nuclei. By this I do not mean merely that they show what I have previously described[2] as the binu-

[1] The smears were fixed with osmic acid vapour, then passed through absolute alcohol, and stained with Giemsa.

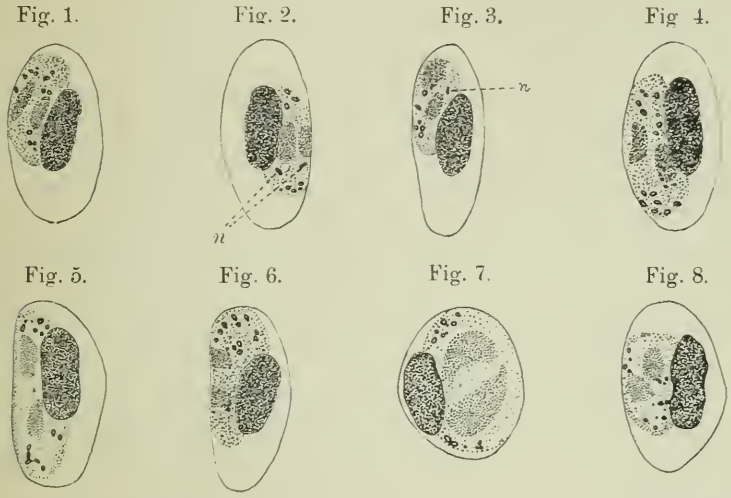
[2] Quart. Journ. Micr. Sci., 53, 1909. p. 339.

cleate condition, but that the large, principal nucleus is double (figs. 1—8). Several of these individuals show the small chromatic element in addition, and in such cases this also is usually double (figs. 2 and 3). The position of the two nuclei vis-à-vis of each other varies considerably, as will be seen from the figures. The line joining them may be approximately parallel either to the short or the long axis of the parasite, or it may run obliquely. In the intermediate-sized forms the body certainly appears single; so far as the cytoplasm is concerned there is not the least indication that two individuals are present. This is the case also in some of the larger parasites (figs. 4—7). Apart from the double character of the nucleus the parasites are typical Halteridia, both as regards general form and contour and the position in the corpuscle. In other large forms, however, the cytoplasm shows a distinct split, usually running more or less longitudinally through the middle of the body. This split is either incomplete (fig. 12) or practically complete (figs. 9, 10); in the latter case the appearance is presented of two individuals, each with a single nucleus, the two parasites lying parallel to each other on the same side of the blood-corpuscle, between the nucleus and the longer edge, i. e. in the customary position.

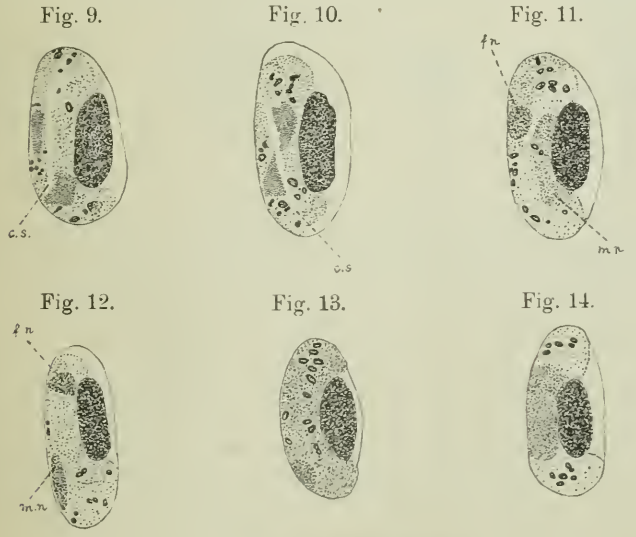
Parasites such as those described occur fairly frequently in the first preparations made. In the next smears prepared, however, five days subsequently, while Halteridia showing this condition are still present, they are much more infrequent — rather scarce, in fact. And in preparations made ten days later again, I have not observed any parasites with the double nucleus.

On first studying this condition, I thought the most likely explanation was that it represented different stages in the binary fission of a single individual. This seemed indicated by the fact that in the intermediate-sized forms, the body appears single, although possessing two nuclei, and only in the large examples is a splitting of the cytoplasm apparent. After prolonged examination of my slides, however, I have come to the conclusion that this cannot be the correct explanation in some cases, at any rate, for I have been able to find two or three parasites in which the double-nuclear condition is present, where one of the two nuclei is distinctly male in character and the other female; i. e., one is large and diffuse and stains bright red, the other small, compact and stains a darker red (figs. 11 and 12). Moreover, the cytoplasm in one part of the body, on the side of the female nucleus, may be denser and stain a somewhat deeper blue that that in the other half of the body, in which the male nucleus lies. The limit or periphery of the body of the parasite is quite even and regular and has the typical contour of an adult ordinary individual. Nevertheless, it is obvious that, in such cases, there

must be really two individuals present, namely gametocytes of opposite sex, which have undergone cytoplasmic union and simulate a single parasite.



Figs. 1—8. *Halteridium fringillae*, from a chaffinch, showing double-nuclear parasites. *n*, chromatic grain (small nuclear element): *p*, pigment. × 2000.
Figs. 2, 3 and 6. Female forms. Figs. 5, 7 and 8. Male forms; the others uncertain.



Figs. 9—14. *Halteridium fringillae*.
Figs. 9 and 10. Parasites showing a distinct split in the cytoplasm (*c. s.*).
Figs. 11 and 12. Parasites in which one of the nuclei is male in character (*m. n.*), the other female (*f. n.*): in the former a distinction in the cytoplasm of the two halves is also apparent. Fig. 13. Adult female gametocyte. Fig. 14. Adult male gametocyte.

Hence it seems to me most probable that the same explanation holds throughout. Of course, where both nuclei are either of male (fig. 7) or else of female character (fig. 6), there is not the same ready means of settling the question as in the above instances. A point which in my opinion also weighs considerably in favour of the view that two individuals are concerned is this. Binary fission of adult or nearly adult sexually differentiated gametocytes (of either sex), to give rise to two intermediate-sized individuals is a phenomenon that has been hitherto quite unknown among Haemosporidia (with one exception, to be mentioned shortly); indeed, I am not aware of anything exactly comparable to such a feature among the Protozoa.

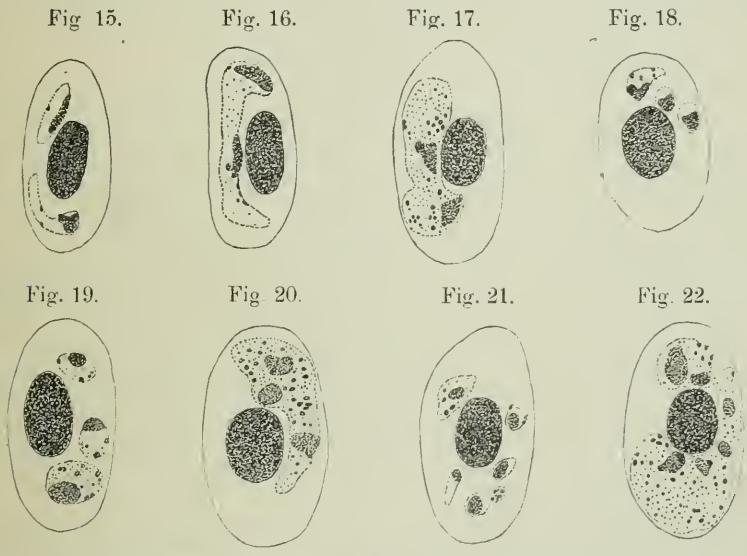
On the other hand, in an ordinary infection (not including in this category an extraordinarily strong one like that discussed immediately), whenever I have observed a red blood-corpuscle invaded by two Halteridial parasites, these have been generally situated either on opposite sides of the host-cell nucleus, or else obliquely, towards the opposite ends. I have only rarely noticed instances where two (and only two) young individuals lie on the same side of the cell-nucleus, and fairly close together.

I may refer here, however, to a case which I observed while at Rovigno, of an extremely heavy infection of a little owl (*Athene noctua*) with *Halteridium* (*Haemoproteus*) *noctuae*, which is most instructive in this connection. In the bird in question, nearly every red corpuscle is infected with the parasites; and in general there are two, three or more individuals — sometimes as many as 6 or 7 small ones[3] — in one corpuscle (cf. figs. 15, 18, 19, 21). In this case it is not at all difficult to find corpuscles containing a cytoplasmic mass which possesses three or four nuclei (fig. 20). Such a condition is the result, I am certain, of the fusion of the cytoplasm of three or four parasites as they have grown in size and come into contact with each other, thus forming a kind of plasmodium (figs. 18—22), which may be indefinite or irregular in form. I cannot consider that such stages should be interpreted in the opposite sense, i. e. as indicative of a schizogonic multiplication. For one thing, the growing plasmodial body contains numerous pigment grains; the young separate individuals in a cell possess little or no pigment. And in spite of the abundance of the material I have not found anything to suggest that such a multinucleate parasite divides up into, or gives off, small uninucleate portions, leaving behind a cytoplasmic residuum containing the pigment. Further, even when there happen to be only two parasites

[3] A similar instance of a multiple infection of a red blood-corpuscle with small Halteridial parasites is figured by Aragao. Arch. Protistenk. 12. 1909. pl. 3, fig. 24.

in a corpuscle, stages can be found showing clearly that as they grow and elongate in the direction of the length of the corpuscle, the two individuals may come into contact (cf. figs. 15 and 16).

It is this feature in regard to which this particular infection of a little owl has an important bearing on the case of the chaffinch-infection under consideration. If fig. 17 is compared with figs. 5 and 6, it will be realized, I think, that the same explanation will apply equally to both cases. If different stages in the relation of two individuals in one corpuscle to each other (such as are drawn in figs. 15 and 16) had been met with alone, it might have been more difficult to decide in which order the series should be taken; but when in the same smear and within a few



Figs. 15—22. *Halteridium* (*Haemoproteus*) *noctuae*, from the blood of a little owl, showing different stages in the growth and cytoplasmic fusion of two or more individuals in a single corpuscle. In the case of these small forms, and particularly where there are three or more parasites in one cell, it is difficult to distinguish whether they are male or female. × 2000.

fields all manner of plasmodial appearances are seen, the result of 3, 4 or more individuals being crowded in a single corpuscle, one is quite unable to regard the process in the former case as binary fission. All things considered, therefore, we must conclude that figs. 1—8 of the condition seen in the chaffinch represent a double infection of a corpuscle, just as it is almost certain that figs. 11, 16 and 17 represent a similar condition.

The interesting and peculiar features about this case, and the reasons which made me hesitate at first as to its true meaning, are these: In the chaffinch in question, the infection is not, comparatively speaking,

a heavy or very abundant one; and I have only seen three or four instances of the ordinary double infection. It is a remarkable fact, besides being of most unusual occurrence, that in such a number of cases two (and only two) small Halteridial individuals should have entered the same corpuscle close together, have undergone cytoplasmic union while young, and assumed just the same form and increased in size as an ordinary single gametocyte (of either sex), ultimately tending to separate as they become adult. I am unable to say, however, whether two such individuals would become, as a rule, fully mature. The great majority of these double parasites which I have seen are nothing like double the size of a ripe gametocyte (cf. figs. 13, 14), hence each half has not nearly attained the maximum size; the parasites of figs. 7 and 11 have as large a bulk as any I have noticed.

In conclusion, I should like to point that what appears to me to be a quite parallel state of affairs in *Haemocystidium* has been described and figured by Dobell[4], though that author has regarded the condition as representing schizogony (in the small forms) or nuclear division (in the adult gametocytes). On the same grounds as those discussed above, I feel fairly certain that Dobell's figures 14 and 16, pl. 7, of very large gametocytes of either sex, each with two nuclei, are really instances of a double infection of a corpuscle by gametocytes of the same sex, just as his figs. 17 and 18 show double infection of a corpuscle with gametocytes of opposite sex. Dobell says that double infection of a corpuscle is not uncommon, but apparently does not take into consideration the possibility that the two individuals in one corpuscle may be of the same sex; and there is no reason to suppose this cannot occur, just as readily as double infection with individuals of opposite sex. Now a corpuscle infected with two gametocytes of the same sex (either male or female), would present the same appearance as shown in Dobell's figs. 16 and 14, respectively; and I have little doubt that is really the condition there represented. Similarly, with regard to the schizogony of small forms, which Dobell says is most usually of the nature of binary fission, I think it is much more probable that the author's figures 4—7 should be read in the opposite direction, as indicating successive stages in the cytoplasmic union of two small individuals which have entered the same corpuscle; granted the occurrence of double infection, the two parasites must enter the cell as small forms. Dobell says the infection is often quite intense; this explains the occasional (infrequent) occurrence of three or four small parasites in a corpuscle, which have united. In short, Dobell's figures are quite comparable to my

[4] Festschrift R. Hertwigs. Bd. I. 1911. p. 123. pl. 7.

figs. 15—20, showing different degrees of infection of the red corpuscles of the little owl with *Halteridium*; and I should say the explanation I have given above applies equally to the case of *Haemocystidium*.

The Lister Institute, August 31st., 1911.