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TEXTBOOK OF AGRICULTURAL BACTERIOLOGY

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PREFACE

THIS "Textbook of Agricultural Bacteriology" was written to give the reader an accurate and fairly complete view of this new and wide field of knowledge. Most of the material presented in the book was collected and used by the senior author while teaching at the University of Leipzig (1903-1914), and under the title "Vorlesungen über landwirtschaftliche Bakteriologie" was published in 1913. Several requests for an English translation have been received since then. It was deemed preferable, however, to await an opportunity when the whole matter could be thoroughly revised and rearranged in such a manner as to make the book most useful for the American and British student. The junior author has used the "Vorlesungen" for his course at the University of Wisconsin, while the senior author's time since 1914 has been devoted exclusively to research work. It is hoped that all the varied experiences incorporated in the book will have added to its usefulness. Inevitably in a joint work of this character there were differences of opinion between the authors on certain points. Inasmuch, however, as the book was designed as a text, it was felt that it would be inadvisable to introduce any evidences of differences of opinion, either by footnote or individual statements.

The first half of the book is devoted to a discussion of fundamental facts, while in the second half the practical application of bacteriology to agriculture has been fully considered. Many problems of great importance to the farmer are treated in the chapters on Dairy and Soil Bacteriology, but their accurate understanding is not assured unless the preceding chapters have also been studied.

The book is neither a laboratory manual nor a reference book, but the quotations given in the text and in footnotes will direct the reader to such literature if this is desired.

We are indebted to Professor E. G. Hastings for valuable criticisms. With a few exceptions the illustrations are originals, mostly made in the senior author's laboratory at Leipzig. About twelve new ones have been prepared by F. L. Goll of the U. S. Department of Agriculture.

WASHINGTON, D. C.
MADISON, WIS.
December, 1922.

F. LÖHNIS.
E. B. FRED.

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TEXTBOOK

OF

AGRICULTURAL BACTERIOLOGY

INTRODUCTION

During the last decades bacteriology has become of great importance to agriculture, because bacteria may act in many ways, both useful and harmful to the farmer. Molds, yeasts, lower algæ, and protozoa frequently participate in such processes. A general term applicable to all these minute living beings is "microorganisms" or "microbes."[1] Accordingly, "microbiology" is another name for this branch of science, and this term is really more nearly accurate than the commonly used denomination "bacteriology." However, the latter expression is fairly well established not only because it has been in vogue for several decades, but also on account of the fact that in most of the processes concerned, bacteria usually exert the greatest influence.

Aims and Scope of Bacteriology.—Bacteria and related microörganisms may participate in the following processes:

1. Human diseases
2. Animal diseases
3. Plant diseases
4. Normal and abnormal alterations of foodstuffs, of milk, butter and cheese.
5. Numerous biochemical processes taking place in sewage, in manure, and in soil.

Bacteria have gained their widest, though unfavorable, reputation as causative agents of *human* and *animal diseases.* Many people consider the term bacteria as practically synonymous with infection, disease, and death. This belief, however, is no less incorrect than it would be to assume that all green plants are dangerous, because a few of them are poisonous. Nevertheless, general interest was first attracted by medical bacteriology, and the discovery of bacteria as causative agents of human and animal contagious diseases made the term bacteriology

[1] Derived from the Greek words μικρός (mikros) small, and βίος (bios) life.

familiar to civilized mankind. Bacteriology soon became an important branch of both human and veterinary medicine and has attained an enormous development within less than half a century.

Plant diseases also have been thoroughly studied for a considerable length of time. In most cases fungi were discovered as causative agents; but more recently some important plant diseases were found to be caused by bacteria. Their study, therefore, has become a part of plant pathology.

Agricultural Bacteriology.—The two main objects of agricultural bacteriology are: (*a*) the study of bacteria and other microörganisms in their relation to foodstuffs, milk, and dairy products, usually called *dairy bacteriology;* and (*b*) the study of the bacterial processes in manure and in soil, usually termed *soil bacteriology.*

The enormous amount of knowledge accumulated with regard to the causative agents of human, animal, and plant diseases, as well as the multitude of problems awaiting further investigation, have made it unavoidable that these subjects were again subdivided among specialists. The same tendency of specialization is also noticeable in agricultural bacteriology. Some laboratories are reserved for dairy bacteriology, others for soil bacteriology, a few for still more specialized work. The thoroughness required for successful research work necessitates far-reaching specialization, but in order to obtain a broad and well balanced knowledge of the whole field of agricultural bacteriology attention should not be centered too much upon certain special problems, although they may at once attract high interest on account of their great practical importance. Sound knowledge of the fundamental facts is the basis upon which all specialization must rest, and the agriculturist who possesses such knowledge will not find it very difficult to gain a correct understanding of new findings in agricultural bacteriology, and to make proper practical application of them, if this is feasible.

Cycle of Matter.—To what extent agriculture and even the continuity of Life itself depend on the incessant and energetic activity of bacteria and related microorganisms can easily be demonstrated. Figure 1 shows in a schematic manner how the eternal cycle of matter is used and regulated by agriculture and industry for the benefit of mankind. The mineral constituents of the *soil* help to build up *plant products.* These may be either directly used as human food, or they may be converted into *animal products,* such as meat and milk, or into *industrial products,* such as linen and cotton garments, vegetable oils, etc. Animal and industrial products again may be either directly used, or they may undergo another transformation before they serve our

purposes; for instance, wool is transformed into cloth, industrial residues like oil cakes are used as fodder, etc. But all these constructive processes would soon come to an end if there were not a complete cycle of matter, that is, if the material used by plants, animals, and men, would not ultimately and regularly return to its origin. All organic residues must again be mineralized, otherwise the earth would long since have been littered with corpses, and all life would have become extinct. It is true that by the respiration of living

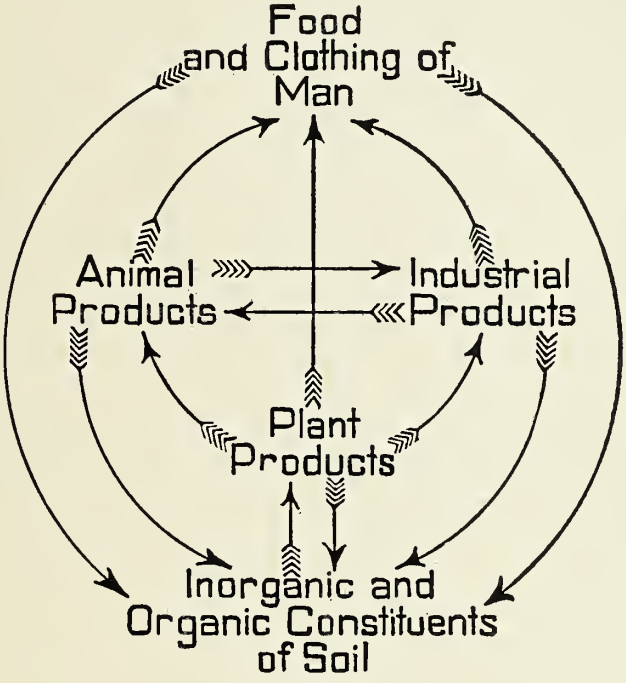

FIG. 1.—Cycle of matter.

plants, animals, and men considerable quantities of organic substances are being constantly broken up into carbon dioxide and water, but this fact does not materially change the general necessity of a permanent equilibrium between constructive and destructive processes, between Life and Death.

Work of Higher and of Lower Organisms.—That the constructive part of the cycle of matter is closely connected with the life of organisms was always self-evident to thinking mankind. On the other hand, only during the last decades was it discovered that nearly every step

in the retrograde transformation of organic substances represents the work of minute organisms, which can not be seen exeept by the use of most powerful optical instruments. Oecasionally purely physieal and ehemical processes participate in these destructive, as well as in the eonstruetive, changes of matter. Nevertheless, the dominating and direeting influenee of living organisms is now equally beyond doubt in both eases. The microorganisms in milk, in butter, in eheese, in manure, and in soil are just as important to the farmer, although he may never see them, as are the milk eows in his stable and the growing erops in his fields.

The task of the medieal bacteriologist usually eenters upon the problem of beeoming aequainted with the disease-producing germ, and to find out how it can be suceessfully fought and eliminated. The farmer, however, should know under what conditions he will be able to secure the most favorable results from the cooperation of the useful bacteria, and how to avoid the detrimental effeets of the aetivities of harmful mieroorganisms. It was an old belief among praetieal agriculturists that barnyard manure adds "life" to the soil, that the surface soil is more active than the "inert" subsoil, that the "ripening" of eream and eheese depends to a great extent on the use of well "working" starters. These and similar expressions indieate elearly how by praetieal experience a fairly correet insight was gained long before exaet baeteriologieal investigations had beeome possible.

Environmental Conditions.—The excellent results obtained by eareful selection and breeding of the cultivated plants and domesticated animals led many to the belief that it should be the foremost task of the agrieultural baeteriologist to seleet and to eultivate the most efficient strains of useful baeteria in order to make them available for the practieal agrieulturist. However, only in a few eases ean such direct results be expected, as for instance in the use of selected bacterial eultures for the preparation of starters in the dairy, or for the inoculation of leguminous seeds. In all other cases the eonditions under whieh these useful microorganisms live and work must first be investigated very thoroughly. *Even the most active bacteria can not display their ability under unfavorable conditions,* just as the best milk eow cannot show a high productivity when improperly kept and fed, nor will the best seed ever produee heavy crops on a badly tilled, weedy soil.

To seeure a eomplete and detailed knowledge of these environmental conditions is by no means an easy, though a very important task of agricultural baeteriology. At the present much remains to be done in this direction, and frequently one must be satisfied if at least the

general principles have been worked out which are governing bacterial life in the different phases of the transformation of matter. Year by year more details will be discovered; but a clear impartial conception of their accuracy and importance will always be dependent on a sound knowledge of the underlying general principles. Therefore these will have to be considered before the various problems of dairy and soil bacteriology can be approached intelligently. A short historical survey of the development of bacteriology will be given first.

Earliest Bacteriological Hypotheses.—A more or less indistinct feeling that many of the processes now known to be caused by bacteria were an expression of some invisible life may be traced back through many centuries. About two thousand years ago an agricultural textbook was written by *Marcus Terentius Varro,* wherein it is emphasized that farm buildings never should be erected on swampy ground. As one of the reasons for this advice the author states that in such land "certain minute invisible animals develop which, transferred by the air, may enter the body through mouth or nose, and may cause serious diseases." In its original form this interesting piece of antique bacteriology reads as follows:[1]

Advertendum etiam, si qua erunt loca palustria, . . . quod in iis crescunt animalia quaedam minuta, quae non possunt oculi consequi, et per aëra intus in corpus per os ac nares perveniunt atque efficiunt difficiles morbos.

It must be left in doubt whether Varro himself was the first to conceive this remarkably accurate idea, or whether he merely copied it from an older unnamed source. *Palladius,* author of another book "On Agriculture," wrote again about 400 years later:[2]

Palus omni modo vitanda est, . . . propter pestilentia vel animalia inimica, quae generat.

(Swamps must be avoided because of the plague or the dangerous animals which develop therein.)

The beneficial effect of the nitrogen fixing bacteria now known to be active in the root nodules of leguminous plants, was also fairly well known among the agricultural writers of ancient Rome. Planting of lupine, vetch, bean, etc., was declared to enrich the soil and to act like an application of barnyard manure. In *Columella's* book "De re rustica," written in the first century of the Christian era, we find lupine, alfalfa, vetch, bean, lentil, chick-pea, and pea enumerated as plants which either enrich

[1] Varronis de re rustica, Lib. I, cap. XII, printed in 1536 by Joannes Gymnicus in Cologne, together with contributions "De re rustica" by Cato, Palladius, and Columella.

[2] Palladii de re rustica,—Lib. I, tit. VII.

the soil or at least preserve its fertility, while all others are said to exhaust the fields.[1]

Earliest Bacteriological Observations.—In numerous mediaeval publications the doctrine of the "contagium animatum" (the living contagion) was treated again and again; certain "animalcula" were supposed to be responsible for various infectious diseases. The first investigators who actually succeeded in seeing bacteria were probably the two Dutchmen *Anthony van Leeuwenhoek* and *Christian Huygens.* Leeuwenhoek himself made the lenses for his manifold studies, upon which he reported in numerous letters to the Royal Society of London. A complete collection of these communications was printed in 1695 at Delft, where he resided, under the title "Arcana Naturae Detecta"

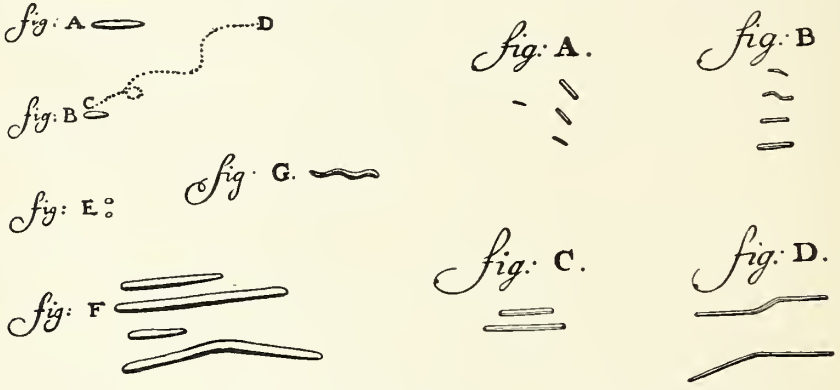

FIG. 2.—Drawings of bacteria made by A. van Leeuwenhoek in 1683 (Figs. *A–G* on left side) and in 1693 (Figs. *A–D* on right side), reproduced in "Arcana Naturae Detecta," 1695, pp. 42 and 335.

(Nature's Secrets Unveiled). The first reference to bacterial life is to be found in a letter dated October 9, 1676, and two very interesting sets of drawings of bacteria were presented in two other letters, written in 1683 and in 1692; both are reproduced in Fig. 2. A few years older than these are some drawings made by Chr. Huygens in a manuscript dating from 1678.[2]

Leeuwenhoek used for his sketches material taken from his teeth, which, as he emphasizes, were perfectly clean and healthy. Some of the bacteria were found to be actively motile, or as the Dutch author says "very gayly moving" under his lenses (indicated by the curved dotted line C-D in Fig. 2). In rainwater and in watery infusions of various

[1] COLUMELLA, Lib. II, cap. X, XI, and XIV.
[2] BEIJERINCK, *Jaarboek der K. Akad.* Amsterdam, 1913.

organic substances he saw similar organisms; but he did not enter into any hypotheses or investigations concerning the rôle these minute "animals" were possibly playing in nature.

Earliest Bacteriological Experiments.—An abstract of Leeuwenhoek's letter of 1683 was published ten years later in the Philosophical Transactions of the Royal Society of London (Vol. XVII, 1693). A few weeks afterwards *Sir Edmond King,* a member of this society, confirmed the correctness of the Dutch author's findings,[1] and also pointed out some important physiological facts which, however, were soon forgotten. To ascertain exactly whether these minute corpuscles were really living beings, he added with a needle small amounts of sulfuric acid, ink, salt, sugar, or fresh blood to the droplets containing the bacteria under his microscope. Sulfuric acid and fresh blood proved to be most injurious; they quickly killed the bacteria, while the other substances

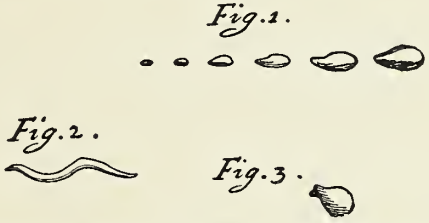

FIG. 3.—Drawings made by Sir Edm. King, Philos. Transact. Roy. Soc. (London) Vol. XVII, 1693, No. 203.

merely caused a temporary shrinking or swelling of the cells. By adding fresh water the original cell form could be reëstablished, provided that the alteration had not gone too far and had not yet caused the death of the organism. This report shows that three facts, generally considered to be quite recent discoveries, i.e., the bactericidal action of blood, the plasmolysis and plasmoptysis of bacterial cells (to be discussed in Chapters I and VII, 6) are clearly described in this early, but long forgotten paper. Some drawings made by King are reproduced in Fig. 3; they are unquestionably inferior to those of Leeuwenhoek.

Earliest Bacteriological Classification.—During the eighteenth century many more or less ingenious speculations were contributed by various authors, but only one real advance in bacteriology was to be recorded. It is represented by the appearance of a beautifully illustrated book on "Infusoria," written by the Danish investigator *O. F. Müller.*[2]

[1] KING, *Phil. Trans. Roy. Soc.,* vol. XVII, 1693, pp. 861–865.
[2] "Animalcula infusoria fluviatilia et marina." Hauniae, 1786.

Several of the generic names introduced by him (Monas, Vibrio, Proteus) are retained up to the present time.

Earliest Practical Application of Bacteriology.—Early in the nineteenth century the first practical results in bacteriology were secured: The Frenchman *Appert* discovered and taught the principles of successfully preserving animal and vegetable foods.[1] A better knowledge of the various possibilities of thorough disinfection was also gained. That in some respects our forefathers had indeed fairly accurate ideas is to be seen, for instance, from what was known at that time about the cause and remedy of the blue discoloration of milk kept in cellars. Some kind of fungus was believed to settle on the surface of the milk; fumigation by burning sulfur and treatment of the vessels with hydrochloric acid were strongly recommended.[2]

In 1837 *Th. Schwann*[3] stated definitely that all fermentative and

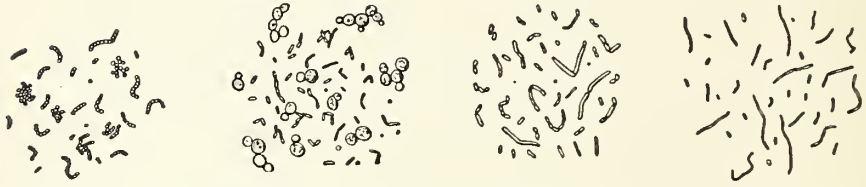

Fig. 4.—Drawings of bacteria published by Pasteur in 1864 (Compt. rend, tome 58, p. 142). (*a*) Urea bacteria. (*b*) Lactic acid bacteria and yeasts. (*c*) and (*d*) Butyric acid bacteria.

putrefactive processes are caused by living organisms ("infusoria" and fungi). Two years later it was emphasized by *Donné*[4] that the alterations in milk should be investigated not only by chemical methods, but under the microscope too. Soon after *C. J. Fuchs*[5] succeeded in clearing up the bacterial causes of the souring as well as of several abnormal changes of milk.

Louis Pasteur and His Contemporaries.—The real foundation of modern microbiology, however, was laid by the famous French chemist, *Louis Pasteur.* Since 1857 he published in the "Comptes rendus de l'Académie des sciences à Paris" numerous papers on fermentation, formation of lactic acid and butyric acid, transformation of urea to ammonia, etc. It is true that his first object was to disprove the old

[1] "L'art de conserver toutes les substances animales et végétales," 1810.

[2] A. Thaer, "Grundsätze der rationellen Landwirtschaft," Bd. 4, 6. Hauptstück, § 54.

[3] *Annalen der Physik und Chemie,* 2. Folge, Bd. 41, p. 184.

[4] *Compt. rend. Acad. Paris,* tome 9, pp. 367, 800.

[5] *Magazin für die gesamte Thierheilkunde,* Bd. 7, 1841, pp. 150, 174, 180–194.

hypothesis of spontaneous generation, which was revived once more at that time, but soon his studies turned to more important and more practical problems, and they stimulated effectively similar research work, especially in France and in England.

An immediate practical application of Pasteur's discoveries to agricultural problems was advocated as early as 1862 in a booklet written by a German farmer named *W. Kette.*[1] It was emphasized therein that in addition to the chemical points of view, as taught at that time by J. Liebig and his disciples, the biological aspects also should be considered, especially with regard to the effect of stable manure and green manures upon the tilth of the soil. Decades passed, however, before this advice was heeded.

Development of Dairy Bacteriology.—More rapid progress was made in the microbiology of milk and dairy products. For example, *von Hessling*[2] wrote in 1866 quite positively that as the various fermentations in milk so also the ripening of cheese is caused by lower fungi; and in a book entitled "Études sur la fabrication de fromage," published in 1867 by *L. H. de Martin,* the differences among the various kinds of cheese were explained as the results of the activity of different species or of various varieties of microorganisms. The intelligent use of starters for cream and cheese ripening was explained and recommended in several books of that time. More detailed information concerning the bacteria connected with the ripening of cheese was sought and secured by *E. Duclaux* in France,[3] and by *Manetti* and *Musso* in Italy,[4] while the famous British surgeon *John Lister*[5] worked on the problem of excluding all bacteria from the milk by observing the greatest cleanliness in every respect. Soon after, in 1884, another Englishman, *O. Ernest Pohl,* made use of these investigations and was indeed able to produce on his farm milk of very low germ content[6] by anticipating those methods which are now recommended by the American Medical Milk Commissions for the production of certified milk.

Development of General and of Soil Bacteriology.—Very thorough botanical investigations upon the morphology and physiology of the bacteria were started in 1872 by *Ferdinand Cohn* at the University of Breslau,[7] and it was in this laboratory that *Robert Koch* developed

[1] "Die Fermentationstheorie gegenüber der Humus-, Mineral- und Stickstofftheorie." Berlin, 1862.

[2] Virchow's *Archiv f. pathol. Anatomie,* Bd. 35, p. 561.

[3] *Ann. agronomiques,* 1879, *Ann. de l'Institut national agromique,* 1879–80.

[4] *Landw. Versuchsstationen,* Bd. 21, 1878, p. 224.

[5] *Quarterly Jour. of Microscopical Science,* New Series, vol. 18, 1878, p. 179.

[6] Helbig, *Pharmazeutische Zentralhalle,* Bd. 51, 1910, p. 1051.

[7] *Beiträge zur Biologie der Pflanzen,* 1872–1876.

his ingenious methods which became the basis of modern bacteriology. In 1876 his classical work on the anthrax bacillus was published [1]; a few years later he also completed the first extensive work on the bacterial content of the soil.[2] Prior to these studies, however, were the very thorough investigations on nitrification in soil, made by the French chemists *Th. Schlösing* and *A. Müntz*,[3] which were later confirmed and extended at the Rothamsted laboratory in England by *R. Warington*,[4] who also worked on denitrification and other important biological processes taking place in the soil.

Discoveries on Nitrogen Fixation.—At Rothamsted, as at other places, interesting data had been collected with regard to the fixation of nitrogen by leguminous plants, but it remained for the German chemists *Hellriegel* and *Wilfarth* [5] to secure complete and final proof that again bacteria, living in the nodules peculiar to the roots of these plants, are directly connected with this process. In 1888 the Dutch bacteriologist *M. W. Beijerinck* succeeded in obtaining pure cultures of these organisms, and soon after he was able to show that they indeed are the causes of root nodules and nitrogen fixation.[6] Until 1921, Beijerinck continued to work at Delft (the same ancient town where more than 200 years earlier Leeuwenhoek had made his first contributions to bacteriology), and many discoveries of great importance to agricultural bacteriology have originated in his laboratory. Best known among them is perhaps his work on Azotobacter, the most vigorous of the numerous bacteria capable of enriching the soil by the fixation of atmospheric nitrogen.[7] Other nitrogen assimilating bacteria had been cultivated before by *Marcellin Berthelot* [8] in France, and by *S. Winogradsky* [9] in Russia. The latter also succeeded for the first time in the difficult task of growing pure cultures of the nitrifying organisms.[10]

Present Status of Medical and of Agricultural Bacteriology.—After Robert Koch and his disciples had solved, in the early eighties of the

[1] In Cohn's *Beiträgen zur Biologie der Pflanzen*, Bd. 2, Heft 2, p. 277.

[2] *Mitteilungen aus dem Kaiserl. Gesundheits-Amte*, Bd. 1, 1881, p. 34.

[3] *Compt rend. Acad. Paris*, tome 77, 1873, tome 84 and 85, 1877, tome 86, 1878, and tome 89, 1879.

[4] U. S. Dept. Agr., *Exp. Sta. Bull.* 8, 1892.

[5] *Landw. Versuchsstationen*, Bd. 33, 1886, Bd. 34, 1887, and "Untersuchungen über die Stickstoffnahrung der Gramineen und Leguminosen," *Beilageheft z. Zeitschr. d. Vereins f. Rübenzuckerindustrie*, Nov., 1888.

[6] *Botanische Zeitung*, Bd. 46, 1888, Bd. 48, 1890.

[7] *Centralbl. f. Bakt.*, II. Abt., Bd. 7, 1901, p. 567.

[8] *Compt. rend. Acad. Paris*, tome 116, 1893, p. 843.

[9] *Compt. rend. Acad. Paris*, tome 116, 1893, p. 1385.

[10] *Annales de l'Institut Pasteur*, tome 5, 1891; *Archives des sciences biologiques*, St. Pétersbourg, tome 1, 1892.

last century, the old questions concerning the causative agents of such dreaded diseases as cholera, tuberculosis, typhoid, etc., the medical branch of bacteriology spread rapidly to all civilized nations, and laboratories for medical bacteriology were established everywhere. With agricultural bacteriology, progress was slower. In Germany, the one-sided chemical point of view, as established by J. Liebig, remained predominant. In France, in England, and in other European countries the investigators were not numerous enough to exert a marked influence. So it became the opportunity of America to offer a promising field to bacteriological investigators. In addition to numerous research laboratories for medical bacteriology, the agricultural branch of this science is equally well represented at the American agricultural experiment stations. During the last decades much progress has been made in this country, and at present about 1000 workers are united in the "Society of American Bacteriologists." If the opportunities offered are adequately used, many valuable results may be expected, because many problems are awaiting thorough investigation.

Literature.—A classified list of books and periodicals devoted wholly or in part to agricultural bacteriology is given below. The large reference books, mentioned under C, will furnish information on special subjects.

A. *Textbooks on General Bacteriology*

W. Benecke, Bau und Leben der Bakterien, 1912.
E. O. Jordan, Textbook of General Bacteriology, 1922.
W. Kruse, Allgemeine Mikrobiologie, 1910.
E. Macé, Traité de Microbiologie, 1912–1913.
Ch. Marshall, Microbiology, 1921.

B. *Textbooks on Agricultural Bacteriology*

H. W. Conn, Agricultural Bacteriology, 1918.
J. E. Greaves, Agricultural Bacteriology, 1922.
E. Kayser, Microbiologie agricole, 1921.
S. Orla-Jensen, Dairy Bacteriology, 1921.
E. Pantanelli, Prinzipali Fermentazioni dei Prodotti Agrari, 1912.
J. Percival, Agricultural Bacteriology, 1920.
H. L. Russell and E. G. Hastings, Agricultural Bacteriology, 1921.

C. *Reference Books on General and Agricultural Bacteriology*

E. Duclaux, Traité de Microbiologie, 1898–1901.
F. Lafar, Handbuch der Technischen Mykologie, 1903–1915.
F. Löhnis, Handbuch der Landwirtschaftlichen Bakteriologie, 1910.
Gino de Rossi, Microbiologia Agraria e Tecnica, 1921–1922.

D. *Books on Bacteriological Technique and Diagnostics.*

F. D. Chester, Manual of Determinative Bacteriology, 1901.
E. B. Fred, Laboratory Manual of Soil Bacteriology, 1916.
C. Günther, Einführung in das Studium der Bakteriologie, 1906.

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K. B. Lehmann und R. O. Neumann, Bakteriologische Diagnostik, 1920.
F. Löhnis, Laboratory Methods in Agricultural Bacteriology, 1913.

E. *Periodicals*

Abstracts of Bacteriology.
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Part I

GENERAL MORPHOLOGY AND PHYSIOLOGY OF BACTERIA AND RELATED MICROORGANISMS

CHAPTER I

MORPHOLOGY OF BACTERIA AND RELATED MICROORGANISMS

Morphological and physiological characters of cultivated plants and domesticated animals determine the degree of usefulness of these organisms. Therefore such knowledge is of fundamental importance to the agriculturist. The same holds true with regard to bacteria and other microorganisms useful or harmful to agriculture. Because most of these organisms can be seen clearly only with a very powerful microscope, a discussion of their morphological features will help in gaining an accurate understanding of their peculiar nature, which is at the root of their surprisingly great activity.

Form and Size of Cells.—While all higher plants and animals represent very complicated and finely adjusted structures of cells and cell compounds, it is the single cell that acts as the living unit as far as bacteria, yeasts, molds, and protozoa are concerned. When these single cells grow and multiply, it often happens, of course, that temporarily a number of cells will be more or less closely connected. Especially the lower fungi (molds) frequently form threads or chains of cells, which sometimes may be seen with the naked eye. But here again the single cell remains the living unit; long threads break up into short joints, so-called *oidia,* which process can be clearly observed with the common white mold (*Oidium lactis*) frequently visible as a white fur-like cover on sour cream.

The most characteristic forms of bacteria, lower fungi, and protozoa are pictured on Plate I. The shape of the single cell is fundamentally the same as in higher organisms: *globular, oval, cylindrical,* or *spiral.* There are smaller or larger differences and variations in every case; and intermediate shapes between ovals and short rod-forms, as well as between cylindrical and spiral cells, so-called comma-shaped organisms, are not infrequent. Generally the cells of yeasts, molds, and protozoa are considerably larger than the bacteria, but also this rule has its exceptions. Azotobacter, shown in Fig. 5, Plate I, one of the most important soil organisms, reaches, for instance, a rather conspicuous size, while sometimes yeasts and protozoa may remain much smaller. Usually special treatments—staining with aniline dyes, or mixing the unstained cells

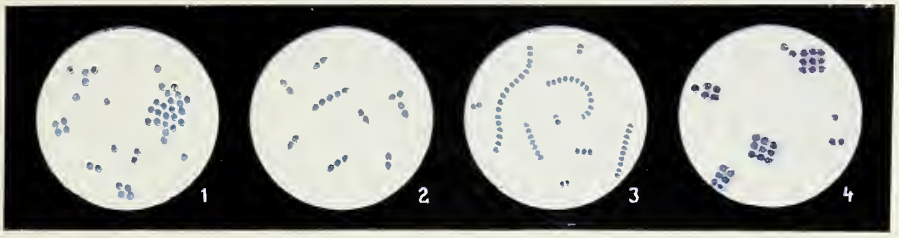
with India ink—are applied to get a clearer picture than is obtainable with the living cells suspended in water.

How incredibly small bacteria really are, will become clear from the following consideration. At 1000-fold magnification many of the rod-shaped bacteria measure about 0.5 by 1.75 mm. A man magnified on the same scale would appear as a giant 1700 meters tall and 500 meters broad. Between such an immense being and men of normal size exactly the same relation in size would exist as between men and the bacteria 1000-fold magnified. Therefore, to reach an accurate conception of the real size of the bacteria, another step of the same relation would have to be made, but this is almost beyond imagination.

Measuring Bacteria.—On account of their minute size bacteria and other microorganisms are measured by "micro-millimeters." One micro-millimeter, usually abbreviated "*micron*" (plur. *micra*) and written 1μ, is equivalent to 1/1000 mm. Most of the globular bacteria, usually called *cocci*,[1] have a diameter of about 1μ. The short rod forms, as they are found, for instance, in the root nodules of leguminous plants, measure usually $\frac{1}{2}$–$\frac{3}{4} \times 1$–$1\frac{1}{2}\mu$, while the long rods reach 4 to 6μ or more in length. Among all the bacteria thriving in milk, butter, cheese, manure, and soil, only a few will be found smaller than $\frac{1}{4}\mu$ or larger than 10μ. Instances of exceptionally large bacteria are found in the case of organisms connected with the transformation of sulfur compounds (Chapter VII, 5); their length may reach 40 to 60μ or more. The cells of the causative agent of pleuropneumonia of cattle, on the other hand, measure only 0.1 to 0.2μ. After the ultra-microscope was discovered, some authors were of the opinion that they had found a special group of "ultra-microorganisms," much smaller than the smallest bacteria known. Undoubtedly such very minute forms exist, but it seems as if they are merely peculiar growth types of larger bacteria or of protozoa.

Size and Efficiency.—Single cells of yeasts, molds, and protozoa are usually 5- to 10- to 20-fold larger than bacteria. For two reasons these differences are of great physiological importance. First, the smaller a body, the greater the area of its surface in relation to its volume. Second, as the exchange of substances in most of the metabolic processes, caused by bacteria or fungi, takes place through the cell wall, the relative size of its surface naturally determines to a large extent the efficiency of the active cell. Figure 5 shows why the smaller size of the bacteria

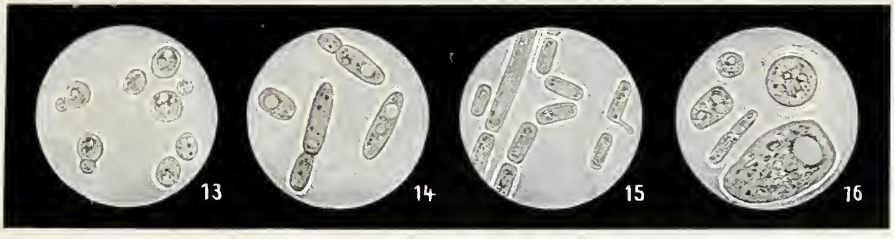
[1] Derived from ὁ κόκκος (kokkos) = fruit kernel. Strictly taken, the term globular bacteria should not be used, because the name bacterium comes from βάκτρον (baktron) or βακτηρία (bakteria), Greek words for rod. But at present the term bacteria is so generally used for all of these organisms, quite irrespective of their shape, that it has practically lost its original meaning.



1-4. Globular bacteria, stained with methylene blue, × 1000
1. Micrococci 2.-3. Streptococci from milk 4. Sarcina

5-8. Rod-shaped bacteria, stained with fuchsin, × 1000
5. Azotobacter 6. Nodule bacteria 7. Bact. casei 8. Hay bacillus

9-12. Curved and spiral bacteria, India ink preparations, × 1000
9. Proteus 10. Vibrio sp. 11. Spirillum sp. 12. Spirochaeta sp.



13-16. Yeasts, molds, and protozoa, living, × 1000
13-14. Yeasts of different shape 15. Oidium lactis 16. Protozoa

renders them more efficient than are the larger fungus cells. Rectangle A represents the surface of a cube whose length of edge is 1 mm. If the calculation is simplified by ascribing to bacteria and fungi cubical shape and an average size of $1\mu^3$ and of $10\mu^3$ respectively, it follows that the 1 mm. cube will be filled by 1 million fungus cells or by 1000 million bacteria cells. The total surface of 1 million 10μ cubes is 600 mm.^{2} (rectangle B), that of 1000 million 1μ cubes, however, is 6000 mm.^{2} (rectangle C). A 100- or 1000-fold reduction in size results in a 100- or 1000-fold enlargement of the active surface and also—at least to a certain extent—of the efficiency of these organisms.

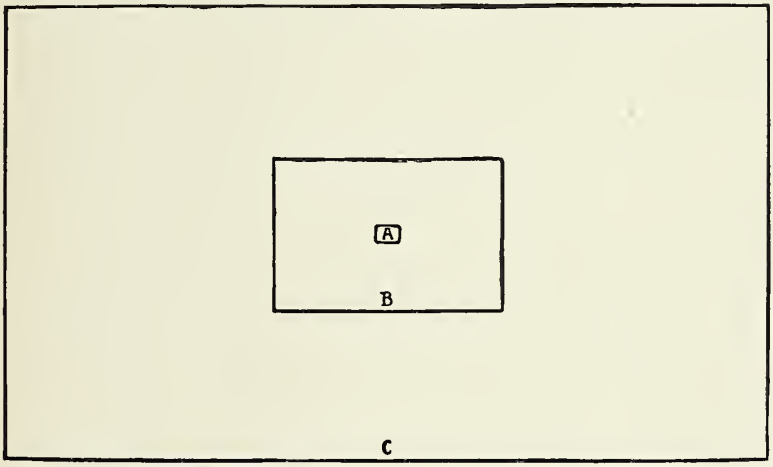


FIG. 5.—Rectangle *A*: Surface of a cube whose length of edge is 1 mm. *B*: Total surface of 1 million cubes whose length of edge is 10μ. *C*: Total surface of 1000 million cubes whose length of edge is 1μ.

One thousand million cells within 1 cubic millimeter, about the size of a pin head, is again something well beyond imagination. If five men would each count two cells per second throughout every one of 300 days in a year, they would finish within this period not more than 100 millions, which is only one-tenth of the total sum.

Size and Number.—Considering the minute size of the bacteria it is easily understood why such large numbers of them may be present in soil, water, air, food, etc. Figure 6 shows three glass containers (1/10 original size) which were filled with 20 kg. milk, butter, and Swiss cheese, respectively. In the centers of the front panes small cubes of black glass were fastened, indicating how much space would be filled by the bacteria present in that amount of milk, butter, or cheese, if they all could be collected in these places.

Wherever bacteria display great activity, rod-shaped cells are most prevalent. This again is easily understood, when it is taken into account that the active surface is comparatively much larger with a rod than with a globule. But globular cells are most frequent among the bacteria in the air; there is little chemical activity, and small globules are naturally better able than rods to float in the air for a long time.

Variability of the Cell Form.—It is a well known fact that the form of higher organisms always varies to some extent, as is especially noticeable with cultivated plants and domesticated animals. Therefore, it is

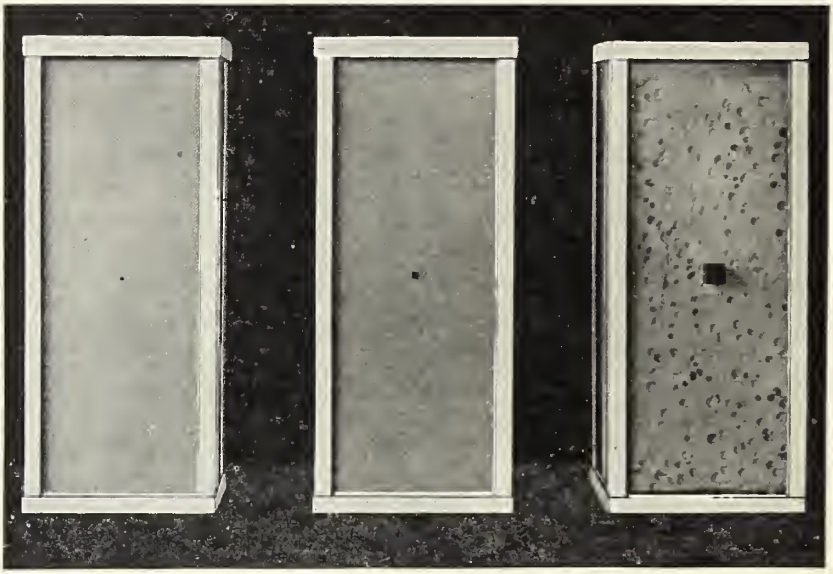


FIG. 6.—Glass containers with milk, butter, and cheese ($\frac{1}{10}$ orig. size). Germ content in milk $2\frac{1}{2}$ millions per c.c.; in butter 20 millions per g.; in cheese 500 millions per g.

not surprising that the small single cells of bacteria and related organisms exhibit similar tendencies. And if one considers how much more they are exposed to the modifying influences of their environment, it will at once become obvious that even much greater variations of the cell forms are to be expected in these cases.

Figure 9 on Plate I presents a bacterium, especially inclined to assume various shapes, which on account of this behavior has been named *Proteus,* in memory of the old Homeric sea-god, of whom it was told that he could change into every form imaginable. But on closer examination the *nodule bacteria* (Fig. 6, Plate I), as well as the *streptococci*[1] taken

[1] A chain of beads, used as necklace, was called by the Greeks στρεπτός (streptos).

from milk (Figs. 2 and 3, Plate I), also display certain variations in their cell forms and the cocci some deviation from the typically globular shape.

But the alterations of bacterial cell forms are not restricted to such comparatively small variations. Figure 1 on Plate II indicates what changes may occur with the slender straight rods of *Bacterium casei,* shown in Fig. 7 on Plate I. Figure 2 on Plate II should be compared with Fig. 6 on Plate 1, and Figs. 3 and 4 on Plate II with Fig. 8 on Plate I (the normal form of *Bacillus Malabarensis* is very similar to that of the hay bacillus).

Involution Forms.—Cells of atypical shape are often termed "involution forms," and this name is indeed quite appropriate as far as such changes are really due to cell degeneration. Involution is contrary to evolution, and synonymous with *degeneration,* that is retrograde development leading to death. Unfortunately, it has become a very widespread habit to speak of involution forms wherever a type of growth becomes visible which the observer considers to be atypical. The opinion that bacteria must be always globules, rods, or spirals, and that only these are typical or "legitimate" forms, has so firmly taken hold of so many bacteriologists that it is usually considered entirely superfluous to make a thorough investigation of the viability and the further behavior of such assumed involution forms. But whenever such investigations were made, it was frequently discovered that these changed cells were by no means pathological or in course of degeneration.

The irregular cell forms of the nodule bacteria appear, for instance, when development is at its height, and they are very active in fixing nitrogen from the air. Other bacteria, participating in the process of nitrogen fixation, display similar changes. And increased knowledge has shown that pathogenic organisms, too, may appear, while fully active. in shapes widely differing from those often called typical.

Monomorphism and Pleomorphism.—After Robert Koch had developed his methods of isolating the bacteria and of growing them in pure cultures, it was soon discovered that under constant conditions a conspicuous uniformity in growth was to be observed. This fact was contrary to earlier opinion. It had been thought before that bacteria, like fungi and protozoa, were able to assume many different forms; they all were considered to be *polymorphous* or *pleomorphous.*[1] But as the bacteria thus far studied were practically all grown in mixed cultures, and the new results, recorded by R. Koch and his pupils, apparently proved without exception that pure cultures of bacteria did not display such

[1] Derived from πολύς (polys) = many, πλέων (pleon) = more, and μορφή (morphe) = form.

pleomorphism, the opposite point of view gained great strength among bacteriologists. The bacteria were now declared to be strictly *monomorphous,* and the theory of monomorphism was taught nearly everywhere; only comparatively few bacteriologists did not accept it.

However, as more and more data accumulated, it became increasingly difficult to reconcile the facts observed with this theory. When the experiments were conducted under strictly uniform and constant conditions, as a rule, uniform and constant results were secured. But exceptions were not entirely absent, and changes in the environmental conditions led to still greater deviations. By declaring one type of growth in each case to be typical, and by discarding other forms as "atypical" or as signs of "involution," the monomorphistic dogma could and can be preserved for a while. But if the facts are weighed impartially, no doubt remains that like lower fungi, algae, and protozoa, which have long been known to be

FIG. 7.—Chain of budding yeast cells (×500).

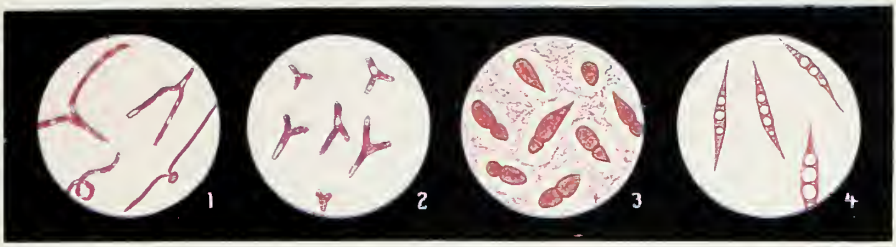
FIG. 8.—Branching thread of a mold (×500).

pleomorphous, the bacteria too are able to assume different cell forms in the course of their full development, although under constant conditions uniformity and constancy are frequently observed.

Cell Compounds; Branched Growth.—With all lower organisms the single cell is the living unit, but cell compounds may be temporarily formed by bacteria, and with the fungi this type of growth is more frequent and more permanent. The globular cells of the cocci may occur in tetrads or in irregular clusters (Fig. 1, Plate I), in which case they are sometimes called *staphylococci,*[1] or in short or long chains as *streptococci* (Figs. 2 and 3, Plate I), or in regular cubical bundles, made up of 8, 16, 32, or more cells (Fig. 4, Plate I), to which the name *Sarcina* is usually applied.[2] Compounds of rod-shaped cells are either chains or threads; in the *chain* the single units are still easily discernible, while in the *threads* the dividing cell walls are less clearly visible or have entirely vanished.

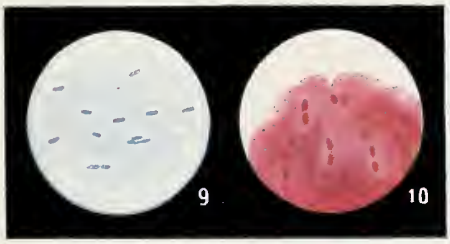
[1] Derived from σταφυλή (staphyle) = bunch of grapes.

[2] Crosswise tied baggage was called *sarcina* by the Romans.

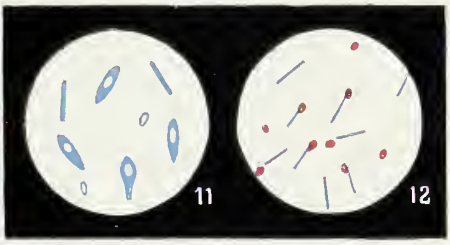


1-4. Branched growth and gonidangia, stained with fuchsin, × 1000
1. Bact. casei 2. Nodule bacteria 3-4. Bacillus Malabarensis

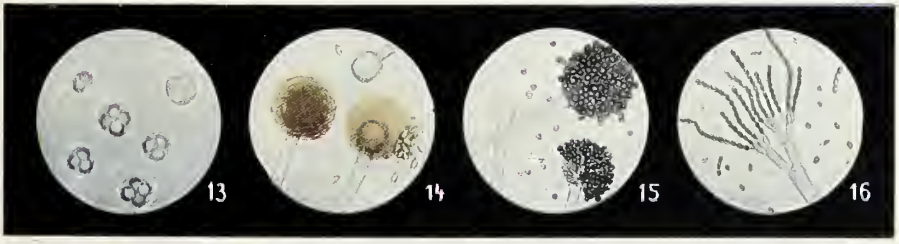
5-8. Bacteria with flagella, stained after Ermengem, × 1000
5. Nitrosomonas 6. B. fluorescens 7. B. radicicola 8. Proteus



9-10. Bacteria with slime
stained, × 1000
9. Nodule bacteria 10. Leuconostoc



11-12. Bacteria with spores
stained, × 1000
11. B. amylobacter 12. B. putrificus



13-16. Yeasts and molds with spores, living
13. Sporulating yeast × 600 14. Mucor × 300 15. Aspergillus × 300 16. Penicillium × 300

Figures 7 and 8 illustrate these differences, and they show at the same time how lateral outgrowth may lead to branched forms.

Figures 1 and 2 on Plate II picture branched bacteria, which according to the monomorphistic doctrine were often classed as involution forms. But sufficient evidence is available at the present time that branching occurs in many bacteria, and very probably in all of them. Even species which usually grow in globular shape may change to irregular branching growth; and it is especially noticeable that branched forms are found more frequently in young than in old cultures, quite contrary to what is to be expected from true involution forms.[1]

Structure of Cells.—The same relation which exists with regard to cell morphology between bacteria, lower fungi, and protozoa on the one side, and the cells of higher organisms on the other, becomes apparent when the inner structure of those cells is examined. Again there is analogy in principle, but greater simplicity in the minute bacterial cells. Their very small size makes such cytological studies, of course, exceptionally difficult, and it is not surprising that the results secured by different investigators are not always in good agreement.[2] The methods used in such studies, especially the various modes of fixing and staining the cells, cause frequently more or less profound alterations of the delicate structures, and vexatious artefacts are often formed. In most cases a highly complicated *protoplasm* represents, in size as in importance, the major part of these minute cells, and this is usually surrounded by a more or less solid *membrane;* only some of the protozoa are without membrane, and therefore characterized by their very changeable forms.

Protoplasm.—Rarely does the protoplasm present a homogeneous appearance; more frequently it is distinctly granular, as shown in Figs. 13 to 16 on Plate I, and in Fig. 7 in the text. Part of these granules are so-called *cell inclusions*, representing either reserve material (fat, glycogen, volutin, etc.) or foreign bodies (for instance bacteria, taken up as food by the protozoa), or they are growing *reproductive* organs, which are liberated when fully developed. Whether or not cell *nuclei* are present among these cell granules, has been a long disputed question, and some authors are still firmly convinced that the bacteria at least have no nuclei. It is beyond dispute that in all microorganisms no such highly organized nuclei are to be found as in the cells of higher plants and animals. But nuclear substances are undoubtedly present, either finely

[1] Detailed references concerning the pleomorphism of the bacteria (also on their branched growth) may be found in F. Löhnis' "Studies upon the Life Cycles of the Bacteria," *Memoirs Nat. Acad.*, vol. XVI, No. 2, 1921.

[2] These cytological problems are thoroughly discussed in A. Meyer, "Die Zelle der Bakterien," and in Ch. Marshall's "Microbiology."

distributed as so-called *chromidia*, or concentrated into one or more globular or rod-shaped bodies. In protozoa and in lower fungi, *vacuoles* are often visible within the protoplasm, while bacterial cells are nearly always completely filled with active protoplasm, which fact furnishes another reason for their comparatively very high efficiency.

Cell Wall.—The membrane surrounding the protoplasm is always more or less slimy, and agglomerates of bacteria are therefore, as a rule, soft, viscous, and sometimes very sticky. Such slimy bacteria prove occasionally troublesome in milk, bread, and in sugar solutions. When examined under the microscope in living condition such cells are surrounded by a bright halo of varying thickness (Figs. 13 to 16, Plate I), while drying and staining usually furnishes pictures like Figs. 3 and 9 on Plate II. The slime has shrunk in these cases, leaving an empty space around the stained cells; sometimes the cells themselves are found lying outside these empty spaces. If the slime is more solid it will be

FIG. 9.—Anthrax bacilli with capsules (×1000).

stained, too; Fig. 10 on Plate II illustrates such a case, representing the so-called *Leuconostoc*, a slime producing streptococcus, sometimes found in sugar factories. A term frequently used for slimy agglomerates of this kind is "*zoogloea*"; it means really nothing else than "animal slime," and should be avoided.

Sometimes the slimy layer around the bacterial cell becomes very solid and forms a so-called *capsule*, which is accessible only to special staining methods. The presence of such capsules is very characteristic for certain species; it is used for instance in diagnosing anthrax bacilli in the blood of diseased animals (Fig. 9). If bacteria growing in long threads surround themselves with such a solid cover, the term *sheath* is used instead of capsule.

Plasmolysis and Plasmoptysis.—Under normal conditions the protoplasm is firmly pressing against the cell wall, but sudden changes in the concentration of the solution surrounding the cell may cause conspicuous alterations. If there is an increase in osmotic pressure at the outside of the cell, water escapes and the protoplasm shrinks accordingly, loosening

itself from the cell wall. In the opposite case too much water may enter the cell, so that the wall breaks under the strain, and the protoplasm is thrown out as a drop with more or less force. The first process is called plasmolysis, the second one plasmoptysis,[1] and it was mentioned in the historical review that such enforced alterations have been observed very early by Edm. King. More recently both terms have been sometimes used rather incorrectly for explaining various occurrences, which in fact have nothing to do with such purely physical effects.

Motility.—If living bacteria are examined under the microscope, suspended in a drop of water or nutrient solution, some of them show active motility, while others do not. Yeasts and molds are all immotile, protozoa on the other hand nearly always motile, except their resting forms. But it is not always easy to decide accurately whether certain bacteria are actively motile or not. Very small corpuscles of the size of bacteria, as for instance the black particles of India ink suspended in water, exhibit also some locomotion, which however is entirely passive, of course. It is the so-called *Brownian movement* which keeps the molecules of liquids permanently in a state of unrest and also causes a continuous swinging movement of suspended minute bodies. If bacteria of great motility are tested, no error can be made; but with slow moving forms much care is needed to reach a correct conclusion. It is due to this fact that the literature contains many uncertain or incorrect statements with regard to the motility of bacteria. Young cultures naturally are best suited for such tests.

The speed of locomotion varies greatly. Some bacteria travel, as men do, 1 to 1½ their own length per second, others are 5 to 10 times faster; but fast flying birds reach the 50-fold of their own length in the same time. The type of bacterial movement also shows much variation. It may be straight, or wiggling, or gyrating. Short rods often tumble about, appearing temporarily, when in an upright position, like small cocci. Spiral forms are whirling like ships' propellers. The aspect becomes especially lively, when in a drop of putrid liquid (old liquid manure, for instance) the motile bacteria are chased around by bacteria-hunting protozoa.

Flagellation.—The organs of locomotion of the motile bacteria are in nearly all cases very thin, whip-like protrusions of the cell wall, so-called *cilia* or *flagella*. Only some spiral forms (classed as spirochaetes) have instead of flagella an undulating membrane, which serves the same purpose. Because of their extreme thinness bacterial cilia can be clearly seen only after having been treated in some special manner. Generally

[1] Derived from λύειν (lyein) = loosen, and from πτύειν (ptyein) = spit.

the staining of bacterial flagella needs much patience and care, and many special methods have been recommended for this purpose. Well made preparations show that the cilia are to be found either all around the cell, or only at the ends singly or in tufts (Figs. 5 to 8, Plate II). Terms frequently used for these different types of flagellation are *peritrichous, polar* or *cephalotrichous, monotrichous* and *lophotrichous.*[1] Cilia are easily thrown off, they may then agglomerate to wavy braids, which are sometimes mistaken for spirilla or spirochaetes.

Active motility increases, of course, the efficiency of the bacteria. They can quickly travel to places where organic substances await destruction, and even fairly dry soil contains enough water to allow free passage to these minute organisms.

[1] Derived from περὶ (peri) = around, τρίχες (triches) = hairs, κεφαλή (kephale) = head, and λόφος (lophos) = tuft.

CHAPTER II

DEVELOPMENT OF BACTERIA AND RELATED MICRO-ORGANISMS

For propagating higher plants, vegetative parts of the mother plant (cuttings, tubers) may be used, or new growth is secured from seeds, that is from special reproductive organs of sexual origin. The same two ways are open for the lower organisms, although the purely vegetative multiplication of cells is much more frequent. The simple, but efficient structure of the vegetative cells of the bacteria favors this kind of development.

Multiplication of Vegetative Cells.—After a cell has reached its full size, it divides into two cells, these when grown sufficiently, separate into four, four into eight, and so on. Because of this simple fission the bacteria have been called *schizomycetes,* which means fission fungi,[1] although fundamentally the same mode of cell multiplication takes place in all lower as well as in the higher organisms. Quite unique, however, is the rapidity of multiplication of bacterial cells. Under suitable conditions a new fission, or a doubling of the cells takes place after 20 to 30 minutes, and if this would go on in the same manner for a day or two, the following stupendous multiplication would result:

One bacterium would produce	
after 1 hour	4 bacteria
after 2 hours	16 bacteria
after 3 hours	64 bacteria
after 8 hours	65,536 bacteria (in round figures 60,000)
after 15 hours	1000 million = approximately 1 mm.^{3}
after 23 hours	65,000 mm.^{3} = 65 cm.^{3}
after 35 hours	1000 million cm.^{3} = 1000 m.^{3}

Therefore, the *possibility* exists that the progeny of one single bacterial cell represents after 1½ days of steady multiplication a bacterial mass that would fill 200 trucks of 5 tons capacity each. It is self-evident that natural conditions will never allow such excessive multiplication. Lack of food, the detrimental effects of metabolic products, the antagonistic action of other organisms, and various other influences will always

[1] Derived from σχίζειν (schizein) = split, and μύκης (mykes) = fungus.

check this rapid development after a comparatively short time. For a while, however, those theoretieal possibilities may indeed become realities, and this is the reason why occasionally bacterial growth will appear and spread with an almost miraculous speed. Exact determinations have shown, for instanee, that a small number of bacteria planted in milk, aetually increased according to the following scale: [1]

	In 2	3	4	5	6 hours
At 12.5° C.	4-	6-	8-	26-	435-fold
36.0° C.	23-	60-	245-	1830-	3800-fold

With one fission every 30 minutes the multiplication would have been: 16-, 64-, 256-, 1024- and 4096-fold. This shows that milk, kept at high temperature, permits indeed an extremely rapid bacterial growth.

Sooner or later this rapid increase is always followed by an equally rapid *decrease*. The following bacterial counts of a sample of milk may serve as an illustration: [2]

	At the beginning	After 3	6	12	18	24 hours
Bacteria in millions per cc...	0.37	12.75	226	8070	32,243	2286

In the case of *fungi* and *protozoa* eell multiplieation is, as a rule, generally slower, but it may continue for a much longer time. Nevertheless, it is a well-known fact that for instanee food of slightly acid reaction (sour cream, cottage eheese, fruit jam, etc.) may be overrun by molds, especially in warm, moist weather, in a comparatively very short time. A few invisible cells grow up to a mass elearly visible to the naked eye.

Formation of Colonies.—On solid or semi-solid substrates (eheese, sour cream, etc.) baeterial and fungous growths at first appear in the shape of more or less regular eircular dises, which are called eolonies. Colonies of molds are made up of a radiate network of threads, the so-called myeelium,[3] whieh ean be distinguished even by the naked eye from the smooth, paste-like eolonies of yeasts and bacteria. In spoiled jellies especially, the latter are sometimes elearly visible as small, whitish or yellowish, globular or lens-shaped bodies, approximately of the size of millet kernels. Because in such substrates the baeteria are unable to make use of their motility, and accordingly the progeny of one cell will develop to a colony at the plaee where the original germ was loeated, semi-solid transparent jellies have become a very helpful means for eultivating and studying baeteria.

In order to obtain an aecurate knowledge of the nature and aetivity of

[1] Cnopf, *Centralbl. f. Bakt.*, vol. 6, 1889, p. 553.

[2] Budinoff, *Centralbl. f. Bakt.*, II Abt., vol. 34, 1912, p. 177.

[3] Derived from μύκης (mykes) = fungus, and ἥλιος (helios) = sun.

the different bacteria, it is absolutely necessary, of course, to *isolate* them and to investigate each kind separately. It is possible, but very difficult, to pick out single bacterial cells under the microscope. More commonly bacteriologists make use of transparent gelatinous media of such composition that these are solid at low, but liquid at a higher temperature, which, however, is not so high as to kill the bacteria. If these are then evenly distributed in the liquefied material, and this is spread out and solidified in a flat, covered glass dish, usually called *Petri dish*, the ensuing growth presents itself in a manner more or less similar to that pictured in Fig. 1 on Plate III.

The filamentous colonies of molds can easily be distinguished from the more compact, whitish, gray, or yellow colonies of bacteria, some of which have caused a greenish or brown discoloration of the nutrient gelatine. A few colonies have liquefied the substrate around them and are slowly dispersing in the liquid; others are completely imbedded, and appear as those small, whitish, grain-like colonies mentioned before. Colonies of yeasts cannot be differentiated by the naked eye from those of bacteria. The small pinkish colony in the foreground to the right was, for instance, made up of yeast cells.

Microscopic Appearance of Colonies.—At a comparatively low magnification, the differences among the various colonies become much more prominent, as may be seen from Figs. 2 to 5 on Plate III. The coarse granulation of the yeast colony (Fig. 4) is due, of course, to the relatively large size of its cells, and the fine rhizoid threads of the mold (Fig. 5) are equally conspicuous.

If a thin cover glass is slightly pressed for a moment against a bacterial colony, then carefully lifted, and stained according to one of the methods commonly used in the bacteriological laboratory, very instructive *contact-preparates* are obtained, which furnish an accurate picture of the bacteria as they are situated in the colony, because practically all cells from the surface of the colony have stuck to the glass, if there was enough, but not too much pressure.[1]

Variation in Colony Formation.—Giant colonies of bacteria and fungi may be secured if care is taken that each colony is surrounded by a sufficiently large area of nutrient substrate which is kept free from all other growth, as is shown in the small glass containers (so-called Soyka-flasks) pictured in Fig. 6 on Plate III. The concentric growth always

[1] Good photographs of contact preparates of bacterial colonies may be found in a paper by C. Axelrad, *Zeitschr. f. Hyg.*, vol. 44, 1903, p. 477. Detailed discussions of colony formation were published by H. B. Hutchinson, *Centralbl. f. Bakt.* II. Abt., vol. 17, 1906–07, p. 65, and by Fr. Orsós, *Centralbl. f. Bakt.* I. Abt. Orig., vol. 54, 1910, p. 289.

noticeable with such colonies, is clearly represented by the zones visible in the giant colony of *Penicillium*. Similar zones may also be seen with bacterial colonies; changing environmental influences (temperature, light, etc.) are responsible for them. In nature the concentric growth of immense fungous colonies finds its expression in the so-called *fairy rings;* the uneven growth of the grass indicates at such places the progressive development and the following decay of the mycelium in the soil, which then exerts a fertilizing influence.

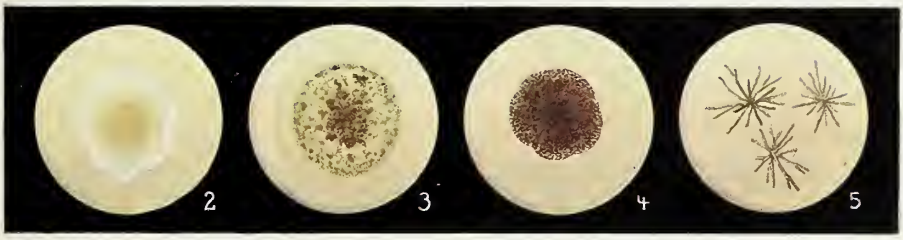
The appearance of bacterial and fungous colonies varies, of course, according to the conditions under which they develop. However, under practically uniform conditions the pictures presented by growing colonies are remarkably constant, characteristic, and of considerable diagnostic value. This regularity and persistence is especially surprising if we keep in mind that with these simple microorganisms the single cell represents an independent living unit. But the same tendency of association which induces bees, ants, and other higher organisms to unite and to enter into complicated, well-characterized organizations, which survive the single organisms, seems to be active even in these very first steps of organic development; the protozoa alone are an exception to this rule.

If the microorganisms are growing in *liquids* the colony formation is less conspicuous, especially with motile bacteria. Nevertheless, also in such cases characteristic agglomerations may become visible, provided that the liquid substrate remains undisturbed. Of special interest are the so-called bacterial "nivcaux" or "plates," which may develop in putrid liquids.[1] In the glass cylinder shown at the left side in Plate IX such a "plate" of sulfur bacteria is seen floating in the middle of the cylinder; curious appendices are hanging down into the lower part of the solution, whose nature will be discussed in Chapter VII, 5.

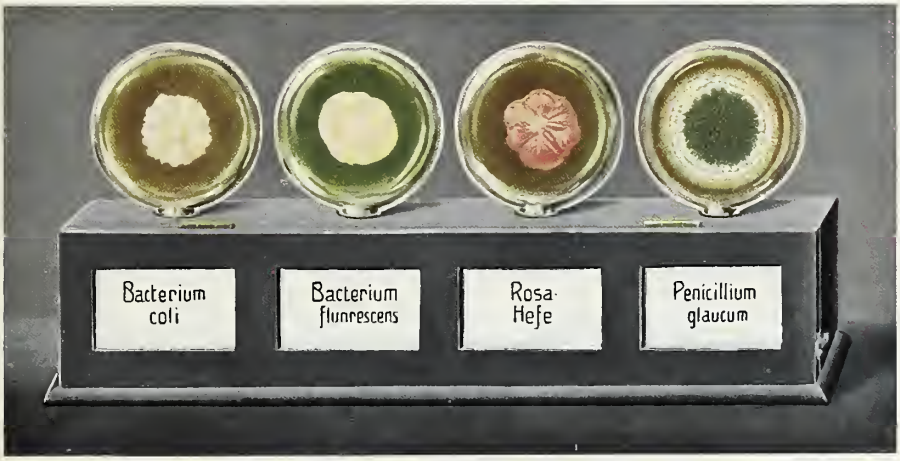
Conjunction, Conjugation, and Copulation.—If well made contact preparates from bacterial colonies are carefully inspected, or if young living bacterial cells are closely examined under the microscope, many cells may be seen which are connected with each other by thin lateral bridges (Fig. 10), or by beak-like protrusions very similar to those occurring with lower fungi and algae. In the latter case it is beyond doubt that a primitive sexual process is taking place between the united cells, which is usually termed conjugation. With the minute bacteria the uniting bridges are, of course, much less conspicuous. For a long time very little attention was paid to these facts, but enough observations are available at present to indicate that this conjunction of bacterial cells is

[1] Beijerinck, *Centralbl. f. Bakt.*, vol. 14, 1893, p. 827; Jegunow, *Centralbl. f. Bakt.* II. Abt., vol. 2, 1896, p. 13; Lehmann und Curchod, l. c. vol. 14, 1905, p. 449.

1. Colonies of bacteria and fungi in Petri dish
$^2/_3$ nat. size



2-5. Microscopic appearance of colonies, × 50
2. Bact. coli 3. B. fluorescens 4. Pink yeast 5. Penicillium



6. Giant colonies of bacteria and fungi in Soyka flasks
$^2/_3$ nat. size

of similar physiological importance as the conjugation or the copulation of other microorganisms.[1] The last named term is usually reserved for those cases where a complete fusion of two cells takes place, as is common especially with protozoa.

Formation of Reproductive Organs.—In the case of higher organisms as a rule sexual processes precede the production of reproductive organs. In principle the same holds true with regard to the lower organisms, but sexual differentiation as well as sexual intercourse is much less conspicuous in this case. Frequently reproductive organs are produced by bacteria, fungi, and by protozoa undoubtedly, or very probably, in an asexual way. Up to the present, many investigators have thought that the reproductive organs of the bacteria were always of asexual origin. The very inconspicuous mode of conjunction was usually

FIG. 10.—Large sulfur bacteria (Chromatium) in conjunction (×750).

FIG. 11.—Sporulating threads of the hay bacillus unstained (living) ×1000.

overlooked. Careful observation reveals, however, that at first (usually during the first four days) many cells are to be found in the conjunct stage, and that the formation of reproductive organs becomes prominent only after this period has passed. Nevertheless, it is not to be doubted that many reproductive organs are produced, indeed, asexually.

Bacterial Endospores.—Best known, most characteristic, and most important are the so-called endospores of the bacteria. They are preeminently resting forms, well fortified against unfavorable influences, and destined to preserve bacterial life over periods of drought, etc., when vegetative life becomes impossible. Old hay, for instance, always contains numerous spores of the so-called hay bacillus (*B. subtilis*). If water is added to the hay the spores germinate to new rods and threads; later new spores are formed therein (Fig. 11), which again survive the dying cells. Except in a few, rather rare cases, only one

[1] LÖHNIS, "Studies upon the Life Cycles, etc.," *Mem. Nat. Acad.*, XVI, No. 2, Chap. IV.

spore is formed in each cell; therefore bacterial endospores do not participate in the multiplication of cells, although such a statement has been made repeatedly. Not all bacteria are able to produce endospores; only a few fairly well characterized groups have this ability, which however may be lost temporarily or permanently.

When the spore is being formed a contraction and concentration of nuclear and cytoplastic material takes place, the newly formed globular or ovoid body surrounds itself with a rather solid membrane, and the rest of the cell gradually fades away. In some cases the diameter of the growing spore is distinetly larger than the width of the mother cell; accordingly, the rod-form of the latter changes to a club-like or drumstiek-like appearance (Figs. 11 and 12, Plate II). Cells of such forms are often called *clostridium* and *plectridium*, respeetively.[1] Due to their low water content, ripe spores are not as easily stained as vegetative cells

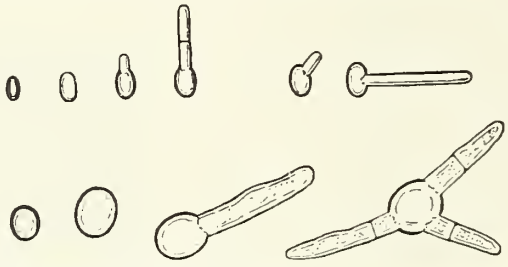


Fig. 12.—Germination of bacterial spores (above) and of mold spores (below).

(Fig. 11, Plate II); many special staining methods have been devised which permit a differential and very characteristie staining of cells and of spores (Fig. 12, Plate II).

The *germination* of these spores usually begins with a more or less pronounced swelling, then the new germ breaks forth either in polar, equatorial, or oblique position (Fig. 12), and the membrane of the spore is either left behind, or it is again (though less frequently) used in making the new cell wall. Some authors believed that the different modes of germination (polar, equatorial, or oblique) could serve for diagnostic purposes. However, they are not sufficiently constant to be accepted as safe marks of distinction.

Other Reproductive Organs of the Bacteria.—Besides endospores four other kinds of reproductive organs are produced by bacteria: Mierocysts, arthrospores, gonidia, and regenerative bodies.[2]

[1] Derived from κλωστήρ (kloster) = spindle, and πλῆκτρον (plektron) = Greek instrument for striking the lyre.

[2] Löhnis, "Memoir," Chap. II.

Microcysts[1] are resting forms, frequently produced by bacteria of globular or oval shape, simply by a thickening and hardening of the cell wall. The Azotobacter cells shown in Fig. 5 on Plate I illustrate this occurrence.

Arthrospores[2] are to be found especially in certain rod-shaped bacteria. These rods divide into several short roundish joints, each of which surrounds itself with a fairly resistant cell wall, and assumes the character of a resting cell.

Gonidia[3] are small round bodies, relatively conspicuous in some large thread-like forms (iron bacteria), but also produced by all other bacteria. In the latter case usually 1 to 4 of them are to be found in each cell; their minute size is responsible for their being very little known. They are not resting forms, but are mostly motile, and may multiply as such before growing up to new regular cells. Therefore, they are able to participate considerably in the multiplication of the bacteria. Frequently they develop while still inclosed in the mother cell, becoming *buds* and *branches* in this case. Occasionally they are produced in greater numbers than four, in which case the mother cell undergoes an inflation and develops to a *gonidangium,* that is a giant cell of globular, club, pear, or spindle-shaped appearance, which was often seen but usually discarded as an involution form (Figs. 3 to 4, Plate II).

Regenerative bodies are also globular, but larger than the gonidia, and have firmer cell walls. When free, they look like micrococci and are able, like these or the gonidia, to multiply as such before reproducing normal cells. They are, in fact, an intermediate step between gonidia and normal cells; sometimes they are also motile. Occasionally they appear exactly like the *zygospores* of fungi and algae at the point where two conjunct cells have united (Fig. 1 on Plate II).

Reproductive Organs of Fungi and of Protozoa.—Among the various reproductive organs of the lower fungi only the following ones may be mentioned here:

Spores, produced within sporangia, as shown in Figs. 13 to 14 on Plate II for a yeast and for a very common mold, named *Mucor,* serving as resting cells as well as for multiplication.

Conidia,[4] produced in great numbers at the end of conidiophores of various shapes, playing an important rôle in the almost ubiquitous distribution of such molds as *Penicillium* and *Aspergillus* (Figs. 15 to 16, Plate II). They cover the mycelia more or less completely (Fig. 6, Plate

[1] Derived from κύστις (kystis) = bag.
[2] Derived from ἄρθρον (arthron) = joint.
[3] Derived from γόνος (gonos) = offspring.
[4] Derived from κονία (konia) = dust.

III), and at the slightest disturbance they rise like a cloud of dust into the air, where they remain floating for a long time. Germination of conidia and mold spores starts as with bacterial spores (Fig. 12), but not infrequently a multiple germination takes place.

Chlamydospores and *gemmae,*[1] formed in somewhat analogous manner to bacterial arthrospores, and like these representing resting forms. When germinating, the chlamydospores produce fertile branches, the gemmae vegetative growth.

The protozoa, too, may either encapsulate, forming a *cyst,* or by segmentation may produce several spores or *sporozoites.* The former are typical resting forms, the latter means of multiplication and reproduction.

Autolysis and Symplastic Stage.—Sooner or later, especially when the food supply becomes insufficient, many cells of bacteria, fungi, and

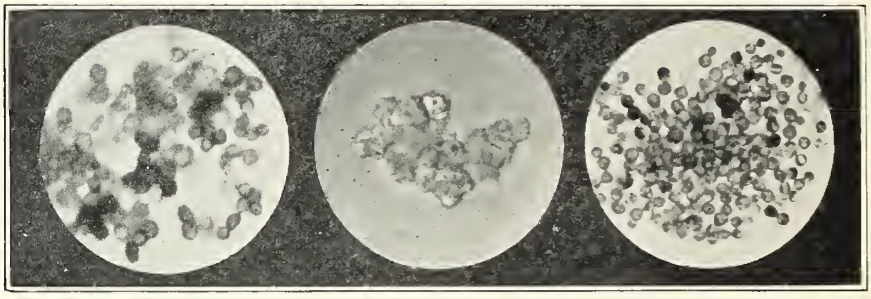


FIG. 13. FIG. 14. FIG. 15.

FIG. 13.—Dissolution of normal Azotobacter cells (×1000).
FIG. 14.—Symplasm (×1000).
FIG. 15.—Regenerative bodies growing from the symplasm (×1000).

protozoa may dissolve, or—according to a frequently used expression—autolysis may take place. This may mean death to the organisms, but by no means always. If the observations are continued, new development may become visible in these amorphous residues, and new cells may be evolved similar to or different from those of the preceding generation. Figures 13 to 15 show these steps in the development of Azotobacter.

If the amorphous product of autolysis is still alive, usually vigorous inner movements are noticeable, and sometimes also a slow, amoeboid, creeping locomotion. Sooner or later minute globoid bodies, so-called *regenerative units,* become distinguishable which either by direct upgrowth or by fusion of several units reproduce new cells. At first regenerative bodies often appear (Fig. 15) like those produced by vegeta-

[1] Derived from χλαμύς (chlamys) = cloak; *gemma* = bud.

tive cells; or besides the normal cells others of various irregular shape may appear, formerly as a rule incorrectly classed as involution forms.

This melting together of the contents of numerous cells, the thorough mixing of the amorphous material, and the re-arrangement of it into new cells are undoubtedly of great importance for the continuity of microbial life in unfavorable environment and for its adaptation to new activity under changed conditions. This phase in the development of bacteria and related microorganisms has been called the *symplastic stage*, and the product of the fusion of the dissolved cells was named *symplasm*.[1] Occasionally the latter assumes globular shape, surrounds itself with a fairly solid membrane, and becomes a *macrocyst*. Such macrocysts have been found with nitrifying bacteria, sulfur bacteria, and others.

[1] Derived from σὺν (syn-, before p: sym-) = together, and πλάσσειν (plassein) = build.

CHAPTER III

CLASSIFICATION OF BACTERIA, FUNGI, AND PROTOZOA

At present bacteria, lower fungi, and protozoa are only partly known. New kinds are discovered nearly every day, but the descriptions given are often very incomplete. Since the middle of the nineteenth century it has been generally acknowledged that the full development, the complete "life cycle," of a fungus or of a protozoon must be thoroughly investigated before such an organism can be properly named and correctly classified. Before that time many so-called species were proposed which later proved to be merely stages in the life cycles of other organisms. Accordingly numerous lower fungi have received several names and have been changed in their systematic positions repeatedly. Due to the monomorphistic dogma, which predominated in bacteriology for several decades, the life cycles of these microorganisms are practically unknown at present, and their classification is, therefore, now in the same position as was that of the lower fungi about fifty years ago.

Artificial and Natural Classification.—When *Linnaeus* began to classify plants and animals upon a scientific basis, he had to rely mostly on more or less complete descriptions of their morphology. In this way a so-called *artificial classification* was secured, which was later replaced by more *natural* classifications founded on a more adequate knowledge of morphology as well as of physiology and of the natural relationship of these organisms. With fungi and protozoa the same change in classifying has taken place more recently, and is still going on. With the bacteria, however, only an artificial classification is possible at present because of lack of knowledge concerning their complete life histories. It is true that besides morphological characters, physiological data are also frequently used in classifying bacteria. Occasionally arrangements obtained in this manner are termed "natural" classifications, but they too are purely artificial.

For agricultural purposes a more practical grouping is usually quite sufficient, viz., to classify bacteria and related microorganisms according to their *activity*, for instance as lactic acid, or butyric acid producing, nitrifying, nitrogen fixing organisms, etc. Undoubtedly this point of view

is of greatest importance to agriculturists, while the other question concerning artificial or natural classification is among the tasks to be solved by the bacteriologists.

Scientific and Common Nomenclature.—According to the rules of scientific nomenclature first promulgated by *Linnaeus* and now generally accepted, each group of organisms of practically uniform character and clearly distinct from others, should receive a double name in Latin, the first word indicating the *genus,* the second one the *species. Triticum sativum* and *Solanum tuberosum* are examples of scientifically correct species denomination.

With respect to microorganisms the situation is much less satisfactory, due (1) to the incomplete knowledge of their characters, (2) to the inclination of some authors to rearrange and rename all that has been classified and named before, (3) to the tendency of many authors to bestow names that are not in accordance with those accepted rules. Sometimes whole descriptions are given instead of binomial names, for instance *Streptococcus acidi paralactici non liquefaciens Halensis,* Hashimoto; *or Granulobacillus saccharobutyricus immobilis liquefaciens,* Grassberger et Schattenfroh. Several authors have renamed bacteria and other microorganisms merely because the new names seemed to them more appropriate. Furthermore, many so-called species have been introduced into the literature despite the absence of adequate descriptions, and the same species name has been repeatedly given to quite different organisms. To-day considerable experience is necessary in order to get a clear view of the whole situation, and much remains to be done before the scientific nomenclature and classification of the bacteria will be in a fairly satisfactory condition.

Besides scientific determinations many common names are in use with lower as well as with higher organisms. The agriculturist speaks of wheat and potatoes, instead of *Triticum sativum* and *Solanum tuberosum,* and in the same manner such common names as lactic acid bacteria, nitrifying bacteria, hay bacillus, tubercle bacillus, etc., are frequently used and quite sufficient in many cases.

Classification of Bacteria.—The following simple arrangement of a comparatively small number of genera has been used by European bacteriologists during the last forty or fifty years:

I. Cells mostly globular, rarely rod-like
- 1. Singly, in pairs, tetrads, or clumps, not in chains. *Micrococcus*
- 2. In pairs or in chains. . . . . *Streptococcus*
- 3. In regular bundles of 8, 16, or more cells. . . . . *Sarcina*

II. Cells mostly rod-like, rarely globular or curved	1. Without endospores	*Bacterium*
	2. With endospores	*Bacillus*
III. Cells mostly curved or spiral, rarely globular or rod-like	1. Of comma-shape	*Vibrio*
	2. Of rigid spiral shape	*Spirillum*
	3. Of flexible spiral shape	*Spirochaeta*

This classification rests entirely on a morphological basis, and on account of the small number of genera many species had to be incorporated into each genus. Nevertheless the system was widely adopted and has proved very helpful. Most thorough work on it was done by *K. B. Lehmann,* professor of hygiene at the University of Würzburg, Germany, who published excellent descriptions of numerous species all based on his own careful experimental studies.[1] Morphological as well as physiological features are both fully considered, and on this basis a clear arrangement into well defined groups was made.

In America not this, but another system of German origin was adopted, proposed about 25 years ago by *W. Migula,*[2] but almost unanimously rejected by the European bacteriologists. Relatively unimportant and highly variable features, such as flagellation, pigmentation, type of germination of spores, etc., were used for defining genera and species. Furthermore, the whole arrangement was based mostly on very incomplete descriptions made by other authors, and only to a very limited extent upon original experimental work.

More recently *S. Orla-Jensen,* professor at Copenhagen, Denmark, proposed another system, which is almost exclusively based on more or less uncertain biochemical facts and hypotheses.[3] Despite the author's claim, it represents by no means a "natural" system. Nevertheless, since the serious defects of Migula's arrangement have led to its abandonment also in America, the proposition made by Orla-Jensen is now looked upon with much favor in this country.

A Committee of the Society of American Bacteriologists adopted several parts of this latest system, combined them with others taken from Migula's and other authors' classifications, and recommended the resulting compilation for general use.[4] The number of genera was considerably increased, but at least most of the families into which these genera were united agree well with the older and better arrangement mentioned

[1] Lehmann und Neumann, "Atlas und Grundriss der Bakteriologie," 6th ed., 1920.

[2] Migula, "System der Bacterien," 1897–1900.

[3] Orla-Jensen, "Das natürliche Bakterien-System," *Centralbl. f. Bakt.,* II. Abt., vol. 22, 1909, pp. 305–346.

[4] C.-E. A. Winslow, J. Broadhurst, R. E. Buchanan, Ch. Krumwiede, Jr., L. A. Rogers and G. H. Smith, "The families and genera of the bacteria," *Jour. of Bact.,* vol. V, No. 3, 1920, pp. 191–229.

above. These are I. Coccaceae, II. Bacteriaceae, III. Bacillaceae, and IV. Spirillaceae. In addition to these, two other families are proposed by the Committee: Pseudomonadaceae (with one genus Pseudomonas Mig.) and Nitrobacteriaceae (with several genera, based on biochemical behavior).

The genus name *Pseudomonas,* originally introduced by Migula and still frequently used by American bacteriologists, was intended to designate cells with polar flagella. It is beyond dispute that the same organism, for instance *Azotobacter,* may have peritrichous or polar flagella, and that closely related species, for instance among the nodule bacteria, may show these two types of flagellation. It was pointed out that this differentiation is especially unsuitable for scientific classification. But as the term Pseudomonas is again revived in the classification proposed by the Committee of the Society of American Bacteriologists, its meaning should be known at least. It remains to be added that the terms *Bacterium* and *Bacillus* are also used in quite a different manner by the followers of Migula: *Bacterium* for immotile rods, *Bacillus* for those with peritrichous flagellation. Occasionally the same generic names find still other application; but this is of little importance in all those cases where the species name is quite distinct, as it always should be. The abbreviation B., as in B. radiciola, B. coli, B. fluorescens, etc., may then mean Bacterium or Bacillus; the species name clearly indicates what organism is meant.

Classification of Lower Fungi.—Only comparatively few groups of lower fungi take part in the processes to be discussed in agricultural bacteriology. They are known as yeasts and as molds.

The *yeasts* (Figs. 13 and 14, Plate I, text Fig. 7) are usually classed as *Saccharomyces* if they produce endospores (Fig. 13, Plate II), as *Torula* if they are non-sporulating, and as *Mycoderma* if they are growing as tough membranes on the surface of nutrient solutions.

Among the so-called *molds* the following five genera are of interest:

Oidium or *Oospora,* characterized by its transparent fragile mycelium, composed of short oidia or conidia (Fig. 15, Plate I); *Oidium lactis* is a most common species, regularly present on sour cream, etc.

Dematium, of similar appearance as Oidium, but with dark colored mycelium.

Mucor, with non-septate, richly branched mycelium, large sporangia (Fig. 14, Plate II), and occasional formation of zygospores.

Aspergillus, with septate mycelium and clubbed conidiophores (Fig. 15, plate II).

Pencillium, with septate mycelium and brush-shaped conidiophores (Fig. 16, Plate II). A rare type of fructification sometimes found with this and the preceding genus is the so-called perithecium (a type of sporangium).

Classification of Protozoa.—Three groups of protozoa are fairly constant inhabitants of water and soil, and occasionally very active as destroyers of bacterial life. They are classed as:

Rhizopoda or *Sarcodina,*[1] motile by pseudopodia. *Amoebae* and *Heliozoa* are wide-spread representatives of this group.

Mastigophora,[2] with long whip-like flagella, of which the Flagellates are most common.

Ciliates or *Infusoria,* covered by a fur of short fine cilia used for locomotion as well as for securing food.

Relation of Bacteria to Fungi and to Protozoa.—Early investigators classed the bacteria because of their motility among the animals (as "animalcula" or "infusoria"). At present they are mostly considered to be plants. In fact, however, neither of the popular terms plant and animal is well applicable to these lowest organisms. They are better left in a class by themselves, or they may be united with all other unicellular organisms under the term *Protista,* as recommended by the well-known zoologist *E. Haeckel.*

Motility and cell structure of the bacteria show many features common with protozoa as well as with lower algae. Other characters resemble those of lower fungi, and since it was discovered that all bacteria are able to grow in branched forms, several authors thought that they should be classed as fungi.[3] There is especially one group, the so-called *Mycobacteriaceae,* showing at least temporarily distinctly fungoid growth, and another one, the *Actinomycetes,* standing exactly on the border-line between bacteria and fungi. Many scientific and practical reasons, however, are clearly against such a merging of bacteria and fungi. It is undoubtedly best to retain the bacteria as a separate group of organisms, showing certain relations to lower fungi, protozoa, and lower algae, but being different from all of them in other respects.

[1] σαρκώδης (sarkodes) means fleshy, referring to their particular appearance. The first name means root-footed (ῥίζα [rhiza] = root, and πούς [pous] = foot).

[2] Derived from μάστιξ (mastix) = whip, flagellum, and φέρειν (pherein) = carry, bear.

[3] E. Bergstrand, "On the Nature of Bacteria," *Jour. Infect. Diseases,* vol. 27 No. 1, 1920, pp. 1–22.

CHAPTER IV

RELATIONS OF MICROORGANISMS TO THEIR ENVIRONMENT

It was pointed out before that the *variability* in form, in motility, and in other characters, so widespread among the bacteria, is largely dependent on the conditions under which they are living. But in nature the environmental conditions are not constant, nor do the organisms always react in the same manner. A more or less speedy *adaptation* to different environmental conditions is frequently noticeable, and in this respect much more profound alterations are possible among the microorganisms, than are known among higher plants and animals. The fundamental reason for this is that within a few days several hundred generations of bacteria may follow each other. Accordingly, changes in the living conditions may lead to conspicuous alterations of the bacterial characters within a few weeks or months, while decades, centuries, or still longer periods would pass, before by similar progressive adaptations of the succeeding generations analogous transformations in the appearance and behavior of higher organisms could be realized. Furthermore, it is easily understood that single cells of relatively simple structure are much more responsive to changed environmental conditions than are complicated organisms, built up of myriads of highly specialized cells.

1. BACTERIAL NUTRITION

Bacteria and other microorganisms obtain their food directly or indirectly by leading either a *saprophytic*[1] *or a parasitic life.* But these differences are by no means constant. So-called parasitic bacteria are cultivated in the laboratory on artificial substrates; and also under natural conditions, they may grow as saprophytes especially in milk and in stable manure. On the other hand, if circumstances are suitable, originally saprophytic organisms may invade living plants and animals and so become parasites.

Some of the bacteria can live, like green plants, on purely inorganic substances, while most of them have to depend on organic nutrients like animals. In the literature the former are not infrequently mentioned as

[1] Derived from σαπρός (sapros) = putrid, and φυτόν (phyton) = plant.

autotrophic or *prototrophic organisms,* the latter as *metatrophic* or *heterotrophic.* But as most, if not all, of the so-called autotrophic bacteria are also able to live in heterotrophic style, it is preferable to avoid these terms as well as those first mentioned.

Chemical Composition of Cells.—The composition of higher organisms varies according to their nutrition. But these variations are again much greater with bacteria than with cells of higher plants and animals.

Vegetative cells of bacteria and fungi contain large amounts of *water* (75 to 98 per cent) like young growing plants. Spores have much less (about 40 per cent), because they consist mostly of concentrated plasmatic substances.[1] Accordingly, the specific weight of bacterial cells is usually close to and a little above 1.0, while that of spores is distinctly higher, approximately 1.3-1.4.[2]

High or low percentages of *mineral substances* are found (2 to 30 per cent in the dried cells) according to the kind of nutrition. What variations are possible in this respect, even with the same species, may be seen from the following data. Cholera bacteria were found to contain in their dry substance:[3]

Per Cent	Grown in Beef Broth	Grown in Mineral Solution
Organic substances { nitrogenous	68	36
Organic substances { nitrogen-free	6	50
Mineral substances	26	14

The *nitrogen content* of bacteria and fungus cells is usually high (about 10 per cent of the dry substance), but sometimes it is found to be as low as 1 per cent. Again the same species may show wide variations; for instance, in one case 1.33 per cent, in another 12.8 per cent N were found in Azotobacter cells.[4] The inclination of this species to produce sometimes large quantities of slimy substances, which are free of nitrogen, was probably the foremost reason for the differences observed. Besides the protoplasm of the cells, the cell walls of bacteria and fungi may also contain nitrogenous substances, among which chitin is best known. "Chromatin" and "volutin" are designations for two groups of nitro-

[1] KRUSE, "Allgemeine Mikrobiologie," 1910, p. 53.

[2] LÖHNIS, "Handbuch der landwirtschaftlichen Bakteriologie," 1910, p. 261.

[3] E. CRAMER, *Archiv. f. Hyg.*, vol. 16, 1893, p. 151; vol. 22, 1895, p. 167.

[4] GERLACH und VOGEL, *Centralbl. f. Bakt.*, II. Abt. Bd. 9, 1902, p. 884; C. HOFFMAN and B. W. HAMMER, *Centralbl. f. Bakt.*, II. Abt. Bd. 28, 1910, p. 137.

genous cell inclusions, whose occurrence is frequently studied in microchemical tests.

Carbohydrates are much less common in the cells of microorganisms than in those of higher plants. Cellulose is mostly absent, and sugar as well as starch is usually replaced by glycogen and granulose. Fatty and waxy substances may be present in considerable quantities, especially in the spores of bacteria and fungi, but also in the vegetative cells of certain species. In the dried cells of the tubercle bacillus, for instance, 40 per cent fat has been found.[1] The presence of fat or wax in the cell walls makes such cells hard to stain. The generally used aqueous staining solutions remain without effect in such cases; this was the reason why the discovery of the tubercle bacilli was not made at an earlier time.

Nitrogen Requirement.—Practically all nitrogenous substances can serve as food for one or another group of bacteria and fungi, which because of this fact play such an important rôle in the transformation of nitrogen in nature.

Generally, *proteins* represent the best sources of nitrogen. Meat, for instance, is very liable to be attacked by myriads of putrefying bacteria; in the laboratory, milk and peptone solutions are used for cultivating numerous species.

Amides and *amino acids* are, as a whole, less suitable, although some of them, especially asparagin and aspartic acid, can also be used in many cases. Others, like urea, uric and hippuric acids, are utilized only by certain groups of bacteria and molds.

Ammonium salts represent fairly good sources of nitrogen for microorganisms, and are superior in this respect to *nitrates*. The assimilation of ammonia by soil organisms is one of the reasons why in fertilizer tests the nitrogen applied as ammonium often does not act as well as does nitrate nitrogen.

Free nitrogen is least suitable and can be assimilated only by a comparatively small number of microorganisms. But even these prefer nitrates and ammonium salts, and some of them grow still more vigorously in the presence of amides and proteins.

Carbon Requirement.—Again practically all carbonaceous compounds occurring in nature may serve as food to some or to many microorganisms.

Proteins can nearly always serve simultaneously as sources of nitrogen as well as of carbon. But some species, for instance certain lactic acid bacteria, are so fastidious that they will grow only if sugar is added to their protein food, as naturally occurs in milk.

[1] Kresling, *Centralbl. f. Bakt.*, I. Abt. Bd. 30, 1901, p. 897.

Among the *carbohydrates* sugars are usually better than starch, and this in turn is generally better than cellulose; but some bacteria prefer the latter very much, and may even be unable to grow in the presence of sugars.

Certain *alcohols,* like glycerol and mannitol, are also widely used, as are many *organic acids* in the form of their salts (malates, lactates, citrates, succinates, etc.).

Carbon dioxide, the main source of carbon for the higher plants, can serve the same purpose with only a few groups of bacteria; and still smaller is the number of microorganisms capable of living on *carbon monoxide* or on *methane,* but these groups are of considerable importance in the regular progress of carbon transformations in nature.

Mineral Requirements.—The same elements which constitute the inorganic parts of the higher organisms, especially phosphorus, potassium, sulfur, iron, calcium, and magnesium, are equally necessary for all microorganisms. Several experiments have been made which seemed to indicate that some of these, namely sulfur, potassium, calcium, and iron, were not essential. But it is next to impossible to exclude the last traces of these elements from vessels and substrates used in such experiments,[1] and a weak growth of bacteria (with about 0.7 per cent total minerals in the fresh substance), weighing only a few thousandths of a milligram, needs such infinitesimal quantities of these elements that the results obtained are never fully convincing. It is certain that in the presence of these elements a better growth was always observed. Especially in regard to phosphorus and calcium it has been noticed repeatedly that the bacterial requirements are sometimes distinctly greater than those of the higher plants;[2] therefore, in addition to the direct effect on the crops the application of calcium phosphates may increase the activity of nitrifying and nitrogen fixing bacteria in the soil, and thus prove helpful in an indirect way.

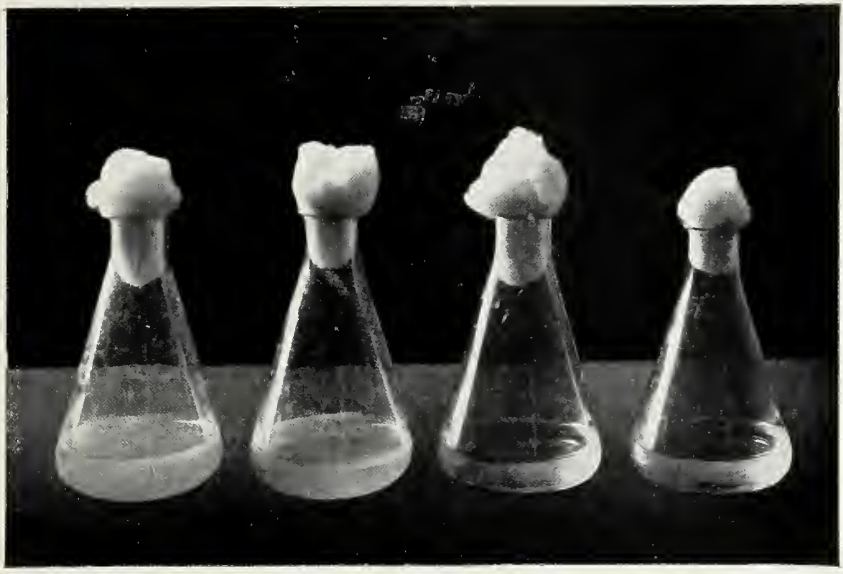
Other mineral elements, especially aluminum, manganese, and silicon, have been also found distinctly beneficial in some cases.[3] They are usually classed as stimulants and will be discussed as such below.

Total Food Supply.—The various inorganic as well as organic nutrients may be more or less readily used according to the combination in which they are offered. Sodium may replace part of the potassium, a rich carbonaceous food supply may increase the availability of a poor

[1] Details are given in a paper by W. Benecke, *Botan. Ztg.* 54, 1896, I. Abt., p. 97; 65, 1907, I. Abt., p. 1.

[2] Löhnis, "Handbuch der landwirtschaftlichen Bakteriologie," 1910, p. 758.

[3] H. Kaserer, *Zeitschr. f. d. landw. Versuchswesen in Oesterreich,* Bd. 14, 1911, p. 97.



1. Development of Bacteria in Tapwater + 0.05% K_2HPO_4
+ 0.5% Peptone, 0.5% Asparagin, 0.5% Ammonium Sulfate, 0.5% Nitrate

2. Growth of Bacteria and Fungi in Tapwater + 0.05% K_2HPO_4
+1% Glycerol and
0.5% Peptone, 0.5% Asparagin, 0.5% Ammonium Sulfate, 0.5% Nitrate

(*Facing page 42*)

nitrogen source, and vice versa. The culture flasks shown in Plate IV illustrate this fact. All of them contained tap water with 0.05 per cent dipotassium-phosphate, to which ½ per cent peptone, asparagin, ammonium sulfate, or nitrate had been added. Those in the lower row received in addition 1 per cent glycerol; small amounts of soil were used for inoculation in all cases. In both rows the peptone and asparagin solutions show good growth of whitish masses of bacteria, while the ammonium sulfate and the nitrate solutions without glycerol remained perfectly clear. In presence of glycerol, however, the development is equal to that in the other flasks. Some floating mold colonies are visible on the ammonium-sulfate glycerol solution.

Bacteria and fungi must have their food in soluble forms, while protozoa can devour solid food. Very different solvents, so-called *enzymes*,[1] are produced by bacteria and fungi in order to make substances accessible to them which are as such insoluble in water. Starch, cellulose, fat, and many other nutrients belong to this class.

The *minimum quantities* of food which still allow bacterial growth, are extremely small. Distilled water, kept in the laboratory for some time, often becomes rich in bacteria and molds. Exact tests of the food requirements of water bacteria[2] have shown that these organisms are sufficiently supplied if they find in 1000 cc. water: 0.002 $\mu\gamma$ dextrose, and 0.00007 $\mu\gamma$ ammonium sulfate ($1\mu\gamma = 1/1000$ mg.); or 1 part dextrose in 500,000 million parts of water, and 1 part ammonium sulfate in 14,000,000 million parts of water. These almost incredibly small figures become intelligible by the following consideration. If 1 cc. water contains 1000 bacterial cells, there are 1 million of them in 1000 cc., which have a weight of approximately 1 $\mu\gamma$ and, accordingly, need 0.002 of their own weight in sugar, and 0.00007 of it in ammonium sulfate. A man is sufficiently supported if he eats daily of carbohydrates 0.005, and of proteins 0.0007 of his own weight, that is, comparatively not much more than those bacteria need.

Stimulants.—It was mentioned above that certain substances, which exert a distinctly favorable influence when present in very small amounts, are usually classed as stimulants, not as food. That is, for instance, the case with manganese. A French author, G. Bertrand[3] pointed out that if only 1 mg. of this element was added to 10 million cc. of nutrient solution, the "stimulating" effect became quite noticeable. However, this quantity is equivalent to 0.1 $\mu\gamma$ per 1000 cc.; in other

[1] Derived from ζύμη (zyme) = ferment.

[2] E. Kohn, *Centralbl. f. Bakt.*, II. Abt. 15, 1906, pp. 690, 777.

[3] G. Bertrand, *Compt. rend. Acad. Paris*, tome 154, 1912, p. 616.

words, the amount of manganese was much larger than that of sugar and ammonium salt in the example just discussed. Many organic and inorganic substances have been classed as stimulants which may, in fact, just as well be considered as accessory food elements. That many of them exert a poisoning influence when present in large quantities would also not militate against this view. It is worth noting that those mineral salts (sodium salts and borates) which contribute to the sterility of part of the alkali soils, act in an analogous manner, viz., favorably in small, unfavorably in large quantities.[1]

Influence of Reaction.—Bacteria prefer generally a neutral or slightly alkaline, yeasts and molds a slightly acid reaction. But this rule again has its exceptions. Lactic, acetic, sulfuric, and other acids are produced by bacteria which can withstand a rather high degree of acidity. But if raw meat is covered with milk in which acid forming bacteria predominate, it is protected against the attacks of putrefying bacteria. The protein substances in cheese are equally protected from putrefaction by the lactic acid first formed. It is well known that accurate control of the acidity of milk, cream, and rennet infusions is of great importance for producing butter and cheese of high quality. Practical experience has discovered that such a control is the best way to regulate the ripening processes, and bacteriology has furnished the explanation and also a more definite knowledge of these facts.

By their own activity bacteria and molds may frequently change the initial reaction. Milk, at first nearly neutral, becomes distinctly acid by the action of bacteria, then molds begin to grow on the surface, consuming the acid and producing alkali. If kept for a long time a distinctly alkaline, brownish liquid of offensive odor results. In soil, where large amounts of proteins are absent, the tendency to acid formation usually prevails; accordingly liming becomes necessary from time to time to neutralize these acids or, as it is sometimes called, to "sweeten" the ground. In other cases increased acidity may prove helpful as a means to suppress the growth of certain detrimental organisms, for instance of those causing "scab" of potatoes.[2]

2. PHYSICAL FACTORS

While in most cases the food requirements of higher plants and animals are distinctly specialized, many microorganisms are known to be able to adapt themselves to more or less different kinds of nutrition. In

[1] C. B. Lipman, *Centralbl. f. Bakt.*, II. Abt. Bd. 32, 1911, p. 58; Bd. 33, 1912, p. 305; Bd. 41, 1914, p. 430.

[2] W. H. Martin, *Soil Science*, vol. IX, 1920, p. 393, XI, 1921, p. 75.

the same manner their relations to various physical factors, as moisture, air, light, etc., are much more varied than those of the higher organisms. Practically every chemical substance occurring in nature can serve as food for one or another group of microorganisms; and even under most extreme physical conditions, where higher plants and animals can not exist, bacteria still continue to live and to perform ceaselessly their work of transformation and destruction.

Water Requirement.—Vegetative bacterial cells contain 75 to 98 per cent water. Accordingly, bacterial life and activity are not possible without sufficient moisture. Protozoa need still more; they are true water organisms. Molds, on the other hand, prefer relatively dry substrates, as is indicated by their growth on stale bread, old leather, etc.

Frequently it depends solely on the water content of a substance whether it will be attacked by molds or by bacteria. Comparatively dry stable manure becomes moldy, but when kept moist, typical putrefaction, due to bacterial action, takes place. Dry parts of silage show mold growth, as does damp hay. If the water content of grain, roughage, or of concentrated animal feed (for instance, in oil cakes and cottonseed meal) is below 12 per cent, they are protected against both molds and bacteria. If more water is present, molds begin to appear, which by their respiration produce carbon dioxide and water; the latter accumulates and increases gradually the original amount until—if time permits—enough water becomes available for bacterial growth and for complete spoilage of the fodder. Materials containing fat are especially liable to undergo such alterations. For instance, powdered rape cake was kept for two years and showed the following composition:[1]

Percentage	Fat		Water	
	Sample I	Sample II	Sample I	Sample II
At the beginning	10.53	8.50	12.45	12.31
At the end	1.98	1.87	21.94	23.42

Normally such material will be used before decomposition can go so far. But this example explains why a moldy odor and even visible mold growth are so common on all foodstuffs not kept perfectly dry.

Soil Moisture.—Soil with 10 to 12 per cent water appears fairly or completely dry to sight and to touch; yet this amount of moisture is usually sufficient for undisturbed bacterial activity. Different soils

[1] H. Ritthausen und Baumann, *Landwirtschaftliche Versuchsstationen*, Bd. 47, 1896, p. 389.

behave differently, and it was reported in the French literature, for instance, that in one soil nitrification took place, although its water content was as low as 7.3 per cent, while in another soil this bacterial process required more than 16.5 per cent.[1] Similar apparently contradictory statements are rather frequent in the literature, but they are due merely to incorrect methods of recording the facts. The amount of water present in a soil should not be given in percentage of the weight of the soil, but in percentage of its water-holding capacity. This is low in sand (usually 10 to 15 per cent of the soil weight), high in loam and clay (about 20 to 30 per cent), and still higher in peat (40 to 60 per cent and more). A saturation of 60 to 80 per cent of the total water-holding capacity of a soil represents the *optimum moisture* for plant growth as well as for normal bacterial action.[2] If less than one half of the water-holding capacity is saturated, plants and bacteria will gradually dry out, but if the water content is very high, only certain plants and bacteria together with the protozoa will persist. Acids and other noxious products accumulate; the soil becomes sour, swampy, and useless for most agricultural purposes if not drained.

Effect of Concentration.—Since the bacteria do not live in chemically pure water, but in more or less concentrated solutions of various substances, the effect exerted by high concentrations sometimes becomes of great importance. In fact, adding large amounts of sugar or salt to substances of high water content is a very old and widely used means of protecting such materials against bacterial invasion. Highly sweetened fruit jams, jellies, and molasses contain so little available moisture that this will suffice only for a moderate growth of molds. Sweetened condensed milk may still contain numerous living bacteria, but they remain inactive on account of the high concentration. Salted meat and fish present other examples of this kind, although the sodium chloride itself exerts its additional detrimental effect, as it does in water or in soil of high salt content. Some bacteria and fungi, however, can endure the strong osmotic pressure exerted by very large amounts of soluble substances. One species (*Bact. vernicosum*), once found in cotton seed meal, was not seriously harmed, for instance, by the following concentrations:[3]

Saccharose	70 per cent,	Lactose	50 per cent,	Sodium chloride	18–20 per cent,
Dextrose	70 per cent,	Glycerin	40 per cent,	Magnesium sulfate	25–28 per cent.

[1] Dehérain, *Compt. rend. Acad. Paris*, tome 125, 1897, p. 282.

[2] Löhnis, "Handbuch der landwirtschaftlichen Bakteriologie," 1910, p. 737.

[3] Zopf, *Beiträge zur Physiologie und Morphologie niederer Organismen*, Bd. 1, 1892, p. 80.

Effect of Drought.—If lack of available water would end bacterial life as quickly as it terminates the existence of higher plants and animals, the metabolic processes caused by bacteria in the soils could not occur with such regularity as they do. Bacteria and fungi, as well as protozoa, are able to produce resting forms which show an increased, sometimes even a surprisingly high resistance against drought and other harmful influences. Nor are the vegetative cells easily killed by lack of water. The slime or the capsules which surround most of these minute cells afford considerable protection. Furthermore, soil which is to all appearances completely dry, still holds small amounts of water in cracks and holes of its inorganic and organic constituents, where numerous bacteria may find a temporary refuge.

If bacteria are dried in very thin layers, as in the dust of rooms and on the streets, the death rate among the vegetative cells is comparatively high. Repeated changes from wet to dry act also in a very unfavorable manner. Quick drying in vacuo, on the other hand, tends to keep cultures alive. But no noticeable activity is to be expected, of course, under such conditions. Rapid drying and storage in dry rooms is, therefore, widely used to protect human and animal foodstuffs cheaply and successfully from the attacks of all kinds of microorganisms.

Oxygen Requirement; Respiration.—All higher organisms need the free oxygen of the air for respiration, that is, for the internal combustion of organic substances, by which means warmth and energy are produced. Many bacteria, fungi, and protozoa breathe in the same manner, but certain groups behave differently. Some oxidize *inorganic* instead of, or as well as, organic substances. Others can live in the presence as well as in the absence of air; and still another group is directly poisoned by free oxygen; it can develop only in the *absence of air,* although a slow and gradual adaptation to the normal mode of respiration has been brought about with some of these organisms.

Figure 16 shows three cylinders partly filled with nutrient gelatin, each of which had been inoculated with different kinds of bacteria. According to their relation to the oxygen of the air, three characteristic types of so-called stab cultures have developed. In cylinder A some whitish growth is visible on and in the upper part of the gelatin, in B the growth along the needle track reaches from top to bottom, but in C some cloudy, flocculent development took place only in the lower third of the substrate.

Anaerobic Life.—Pasteur, who made the first thorough studies upon these different behaviors of the bacteria, named the two groups living respectively in the presence and in the absence of air, "aérobies et

anaérobies."[1] Since then it has become customary to define them as strictly aerobic, or strictly anaerobic, while the intermediate group is usually termed facultative anaerobic or facultative aerobic. Beijerinck[2] is of the opinion that the anaerobic organisms also need a small amount of free oxygen, and calls them therefore microaerophilic. But an exact proof of the

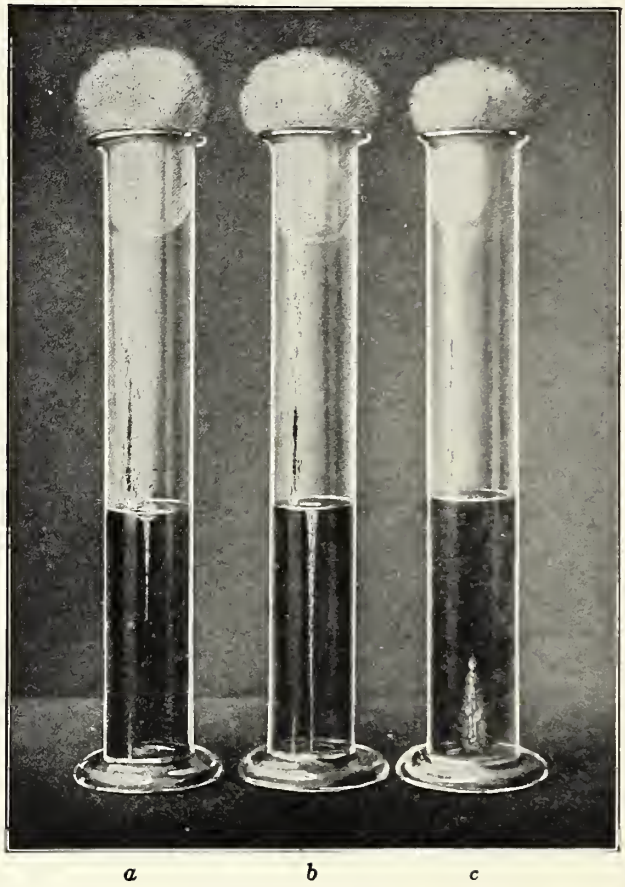


FIG. 16.—Gelatine stab cultures ($\frac{2}{5}$ nat. size). (*a*) aerobic, (*b*) facultative anaerobic, (*c*) strictly anaerobic growth.

correctness of this assumption is lacking, and it is more probable that the contrary view holds true,[3] although a final decision is practically im-

[1] *Compt. rend. Acad. Paris*, tome 56, 1863, p. 1192, note 1. The terms are derived from ἀήρ (aer) = air, and βίος (bios) = life.

[2] *Arch. néerland.*, 2 ser., vol. 2, 1899, p. 397.

[3] BURRI und KÜRSTEINER, *Centralbl. f. Bakt.*, II. Abt. Bd. 21, 1908, p. 289.

possible, because minute traces, hardly detectable even by the most refined chemical methods, may still mean much to the equally minute bacteria, as was discussed in the preceding chapter.

Anaerobic bacteria play a very important rôle in nature. If they were absent, all residues, corpses, etc., buried deep in water-logged ground, would not be decomposed at all, but preserved for centuries. On the surface of the ground, too, the destructive processes would take much more time, if dead bodies, for instance, were attacked only from the outside by aerobic organisms. But by the combined "front and rear attacks" of aerobic and anaerobic bacteria surprisingly rapid destructions are secured.

Oxygen Maxima and Minima.—Special investigations upon the limits of oxygen pressure, endurable to aerobic, facultative anaerobic, and to strictly anaerobic bacteria, respectively, have shown[1] that representatives of the last named group are unable to grow in an atmosphere containing more than 0.13 to 1.04 per cent free oxygen, which is less than 1/100 to 1/10 of the volume usually present in air. Facultative as well as strictly aerobic bacteria have in most cases very low oxygen minima (usually close to 0), while their maxima were found to be between 2 and 6, usually around 3 to 4 atmospheres.[2] Such wide ranges of endurable oxygen concentration are again quite exceptional, but this fact explains why increased pressure of common air has hardly any detrimental effect upon aerobic and facultative anaerobic bacteria. Even a quick increase to 3000 atmospheres pressure proved to be of very little effect, which, however, became quite noticeable if sudden changes of high and low pressure were several times repeated.[3] Pressures of 7000 atmospheres killed vegetative cells of bacteria and fungi after a few minutes.[4]

Oxidation and Reduction.—Anaerobic as well as aerobic life depends, of course, on the continuous oxidation caused by respiration. But while free oxygen from the air is used by the aerobes for this purpose, only compounds rich in oxygen, as sugar, nitrates, sulfates, etc., are used by the strictly anaerobic organisms. The fixed oxygen is taken from these substances, and they undergo therefore more or less far-reaching reductions to methane and hydrogen, nitrites and ammonia, sulfites and sulfides. Because nitrate contains relatively more oxygen than does sugar, it is used to protect the latter against the attacks of facultative

[1] Chudiakow, *Centralbl. f. Bakt.* II. Abt. Bd. 4, 1898, p. 389.
[2] Porodko, *Jahrbücher f. wissenschaftliche Botanik,* Bd. 41, 1904, p. 61.
[3] Chlopin und Tammann, *Zeitschr. f. Hygiene,* Bd. 45, 1903, p. 171.
[4] Hite, Giddings and Weakley, W. Va. Agr. Exp. Stat. *Bull.* 146, 1914.

and strictly anaerobic bacteria in cheese, where in the absence of nitrate the milk sugar might be reduced under liberation of hydrogen, resulting in gassiness of the cheese.

There are numerous other cases where according to circumstances the activities of aerobic or of anaerobic organisms are favored in order to attain the desired oxidation or reduction. Drainage, tillage, and cultivation of the soil assure a fairly thorough aeration leading to the oxidation of organic residues and to the formation of nitrates. In the silo and in rotting manure all practical means are used to insure the exclusion of air and thereby the activity of anaerobic bacteria, which are useful in these cases.

Organic and Inorganic Respiration.—Carbonaceous organic substances, such as sugars, fats, and acids, are the sources of energy in the respiratory processes of most of the lower as well as of all higher organisms. But the intensity of oxidation is again comparatively much greater with the minute bacteria, because of their relatively large active surfaces. Comparative tests have shown, for instance, that the following amounts of *carbon dioxide,* calculated in percentage of the weight of the various organisms, were daily produced:[1]

By beet roots .075 per cent,	By molds 2– 2.7 per cent,
By roots of cereals 1–2 per cent,	By bacteria 7–37.5 per cent,

The intensive carbon dioxide formation steadily going on in silos, in manure, in the soil, etc., is of great importance in many respects, which will be discussed later.

The energy liberated by the respiration of organic substances originates from the sun, and is accumulated by the green plants producing these substances. All organisms living in this manner, and they are by far in the majority, are in fact motors driven by the energy of sunshine. But there are certain groups of bacteria, able to derive their energy from the oxidation of inorganic substances, and therefore to live independent of solar energy. They oxidize *ammonia,* or *hydrogen,* or *hydrogen sulfide,* to nitrous and nitric acid, to water, or to sulfuric acid, respectively. These are processes of great physiological, and in part also of considerable practical importance. Naturally, the oxidation of organic compounds is not entirely absent in these organisms, because their own bodies

[1] These calculations are based on data published by Stoklasa et al. in *Centralbl. f. Bakt.,* II. Abt. Bd. 14, 1905, p. 727; Bd. 29, 1911, pp. 401, 457; *Berichte d. Deutsch. Botan. Gesellschaft,* Bd. 24, 1906, p. 22; *Jahrb. f. wissensch. Botanik,* Bd. 46, 1908, pp. 73, 80; and by Bassalik in *Zeitschr. f. Gärungsphysiologie,* Bd. 2, 1912, p. 27. The results given for dry weight were changed to figures for fresh weight by assuming that beet roots and bacteria contained 15 per cent, roots of cereals 30 per cent dry substance.

are built up of sueh substances. The main souree of energy, however, which also enables them to assimilate carbonic aeid and to produce in complete darkness the organic compounds which they need, is represented by those various modes of "inorganie respiration."

Influence of Temperature.—The vegetative cells of microorganisms are filled with a mixture of protein substances and much water. Because the latter freezes at 0° C. and the former eoagulate at 70° to 80° C., active life is generally limited by these degrees. Some slow aetion at temperatures below 0° C. is occasionally notieeable, either if the eell solution on aceount of its eoncentration does not freeze promptly, or if suffieient enzymes are present to continue the transformations begun at higher temperatures. The gradual deterioration of eold storage butter illustrates these principles. At very low degrees, however, even such activities end, and oecasionally corpses of mammoths have been dug out from solid ice in northern latitudes, so completely preserved during thousands of years that their meat was still readily eaten by the dog teams of the exploring parties.

Slow eooling and slow freezing do not kill the mieroorganisms as a rule. Repeated quick alternations of freezing and thawing, however, prove to be detrimental to lower as well as to higher organisms. Vegetative eells of baeteria and fungi have been kept in liquid air, and even in liquid hydrogen (at—252° C.) without serious harm, provided that the changes from freezing to thawing were not too rapid.[1] Aretic soils contain the same microflora as soils of warmer elimates.[2] And the freezing of milk is without influenee upon the laetic acid bacteria living therein.[3]

Bacterial Life at Low and at High Temperatures.—Certain microorganisms prefer low, others high temperatures. The former are called *psychrophilic* or cryophilic (eold loving), the latter *thermophilic* (warmth loving).

Psychrophilic baeteria are eommon in water of low temperature, they predominate in soil during the eold season, and they multiply quickly in milk and butter kept at low temperature. An increase from 2 to 4500 millions per ee. was observed, for instanee, in milk kept 4 weeks at approximately 0° C.[4] Typieal lactic acid baeteria work very slowly below 10° C.; therefore a more or less abnormal mieroflora develops in milk kept for some time at low degrees, which eauses a deterioration

[1] A. Macfadyean and S. Rowland, *Proc. Roy. Soc.* (London), vol. 66, 1900, pp. 339, 448; vol. 71, 1902, p. 76.

[2] Barthel, *Meddel. om Grönland*, LXIV, 1922.

[3] Bredinow, *Centralbl. f. Bakt.*, II. Abt., Bd. 34, 1912, p. 183.

[4] Löhnis, "Handbuch der landwirtschaftlichen Bakteriologie," 1910, p. 150.

similar to that noticeable in pasteurized milk kept some days at a higher temperature. The characteristic disagreeable flavor often noticeable in food kept a few days in the ice box is also partly caused by such psychrophilic organisms.

In a moderate climate those species predominate in nature which have their temperature minimum close to 0° C., their optimum at 10° to 20° to 30° C., and their maximum in the neighborhood of 40° C. Unfavorable conditions, lack of food, etc., narrow the range of suitable temperatures, while it may expand more or less if other circumstances are very favorable.

Thermophilic bacteria have their minimum at approximately 35° C., their optimum at 50 to 65° C., and their maximum at 75 to 80° C. They are numerous in soils of hot climates, in hot wells, and in loosely packed moist organic substances where temperatures rise quickly, sometimes to the point of ignition (as in damp hay, cotton, horse manure, etc.). The thermophilic bacteria themselves participate by their respiration in the production of heat, and are in turn stimulated by the rising temperature until the maximum is reached. In regard to their active participation they are called *thermogenic* (heat producing).

Influence of Light.—It is a well-known fact that sunlight helps to prevent and to drive out diseases. Contrary to the green plants, which can not live without light, because the chlorophyll action depends on it, most of the bacteria and fungi are hurt or even killed by direct sunlight. Especially the disease producing germs are very sensitive, while other groups are more resistant. The majority of soil, manure, and milk bacteria are practically unaffected by diffuse daylight, but intensive sunshine reduces their numbers quickly if thin layers are exposed. For instance, only 1/5 or 1/6 of the bacteria originally present in a soil sample survived when this was exposed to bright sunshine for five hours in a layer only 1 mm. deep.[1] In thin layers of gravel moistened by dilute urine the following amounts of nitrogen were transformed into nitrate:[2]

	Sample I	Sample II
In the light.....	19 mg.	110 mg.
In darkness.....	86 mg.	360 mg.

In a few cases sunlight acts favorably by stimulating the pigmentation of certain bacteria, and in soil as well as in water the growth of algae is naturally increased. A good growth of soil algae means a better supply of organic substances for the soil bacteria, especially for those fixing

[1] KEDZIOR, *Archiv f. Hyg.*, Bd. 36, 1899, p. 323.
[2] SOYKA, *Zeitsch. f. Biologie*, Bd. 14, 1878, p. 466.

nitrogen from the air. In water the purifying action of algae is added to that of the direct bactericidal effect of sunlight.

The real causes of the detrimental effect of light are not yet fully known; but it is certain that they are not always the same. Physical as well as chemical actions may participate; heating, drying, formation of peroxide of hydrogen and of acids in organic substrates are some of these possibilities. Furthermore, the living protoplasm within the bacterial cell is also directly affected by strong light.

Effects of Other Physical Factors.—*Ultraviolet rays* are still more detrimental than is sunlight; successful application of this fact is made in water purification (see Chapter VI). *X-rays,* on the other hand, have very little or no effect.[1] *Radium rays* are more or less harmful; bacteria so treated become radioactive themselves.[2] *Electric currents* may act favorably or unfavorably, according to circumstances. Physical as well as chemical reactions take place; these, not the electricity as such, are responsible for the results obtained.[3]

Mechanical treatment may also cause widely varying effects. Vigorous shaking, especially in the presence of hard bodies (small glass beads, etc.), naturally destroys the soft cells of bacteria, but reproductive organs, like the minute gonidia, may survive and later produce new vegetative growth. Less severe shaking may merely separate the cells previously united in colonies, and thereby stimulate their multiplication. At the same time a more thorough mixing with the substrate and increased aeration may lead to more vigorous chemical action. For instance, soil samples, after being kept in pots for several weeks, were either left untouched, or were repeatedly stirred during six weeks, and were then tested with regard to nitrification. The following quantities of nitrates were found in every 100 g. of soil:[4]

	Sample I	Sample II	Sample III
Untouched....	2–3 mg.	2 mg.	2 mg.
Stirred.....	39–44 mg.	46–51 mg.	57–71 mg.

Similar though less far-reaching stimulating effects are secured by thorough cultivation of the fields, or by emptying and refilling of pots or pails in greenhouses.

A special influence of gravitation, the so-called *geotropism,* was for-

[1] Beck und Schultz, *Archiv. f. Hyg.*, Bd. 23, 1896, p. 495; Wittlin, *Centralbl. f. Bakt.*, II. Abt., Bd., 2, 1896, p. 676.

[2] W. Hoffmann, *Hygienische Rundschau*, Bd. 13, 1903, p. 913; A. R. Green, *Proc. Roy. Soc.* (London), vol. 73, 1904, p. 375.

[3] Lehmann und Zierler, *Archiv f. Hyg.*, Bd. 46, 1903, p. 221.

[4] Dehérain, *Compt. rend. Acad. Paris*, tome 116, 1893, p. 1094.

merly held responsible for a particular type of growth noticeable with stab cultures of certain species (*Proteus, Bac. mycoides,* etc.) when grown in gelatin. From the needle track fine branches are spreading either horizontally or in an upward direction. This was explained as due to "negative geotropism." But renewed tests have shown that it is the elasticity of the gelatine which decides the length and direction of these lateral branches. The term "elasticotropism" was therefore proposed.[1]

3. SYMBIOSIS AND ANTAGONISM

The majority of microbiological studies is made with pure cultures in the laboratory. Practically all our present knowledge rests on such investigations. Yet despite the very great value of this kind of work, it must be emphasized that much remains to be done before the activities of the microorganisms in nature are fully understood and explained. Associations of many different species are nearly always at work under natural conditions, and the environmental conditions themselves are more or less different from those which can be duplicated in the laboratory. If only experiments with pure cultures are made "our conclusions might be"—according to a very proper remark made by Dr. Chas. E. Marshall[2]—"like studying man apart from society in order to obtain his social relations." But as the behaviors and activities of microorganisms show much more variation and differentiation than is known among higher plants and animals, there are also, of course, many more possibilities of helpful cooperation or of vigorous competition in the struggle for life.

Symbiosis.—An especially interesting example of mutually helpful cooperation, usually called symbiosis,[3] is furnished by the leguminous plants and the bacteria living in their root-nodules. The former supply the latter with large quantities of carbohydrates, and receive in turn all the nitrogen they need even in a soil devoid of nitrogen compounds, and both symbionts are evidently greatly benefited by this exchange. Practically the same correlation may become active between green algae and nitrogen fixing bacteria in water as well as in soil. In water especially so much organic matter can be produced by this symbiotic process that a considerable amount of fish food is derived from this source.[4] Besides these, there are numerous other possibilities for acquiring a larger amount

[1] H. Zikes, *Centralbl. f. Bakt.*, II. Abt., Bd. 11, 1903, p. 59; H. C. Jacobsen, l. c. Bd. 17, 1906, p. 53; H. Kufferath, *Annales de l'Institut Pasteur,* tome 25, 1911, p. 601.

[2] *Centralbl. f. Bakt.*, II. Abt., Bd., 11, 1904, p. 740.

[3] The Greek word συμβίωσις (symbiosis) means "living together."

[4] Hermann Fischer, *Centralbl. f. Bakt.*, II. Abt., Bd. 46, 1916, pp. 304–320.

or a more suitable kind of food by cooperative action. Of special interest in this respect is the fact that new bacterial development as a rule proceeds with much more vigor and rapidity when it starts from a considerable number of cells of the same kind, not from a solitary cell. The stimulating effect of such an association is also noticeable when most of these cells are dead. The components of their bodies improve the food supply of the surviving cells.

Alteration of the *reaction* of the substrates plays also an important rôle in symbiosis. In milk and in cheese the activity of lactic acid bacteria stimulates the growth of various molds, which in turn destroy the acids formed and re-establish an alkaline reaction, thereby favoring again bacterial growth. Many symbiotic actions contribute to the ripening of cheese, and it has been noticed repeatedly in the course of such experiments that pure cultures which remained more or less inactive when tested separately, developed a vigorous and characteristic action if properly mixed.

Of exceptional importance is the symbiosis of *aerobic and anaerobic bacteria* in nature. The extreme sensitiveness of anaerobes against free oxygen would preclude their existence and activity nearly everywhere, if it were not that the presence of aerobic bacteria affords protection. The intensive respiration of the aerobes removes the oxygen from the air in the immediate neighborhood, replacing it by carbon dioxide; other metabolic products may add to the beneficial effect of this symbiotic action. After numerous anaerobic cells have grown they themselves are able to protect the younger cells in an analogous manner. Well developed cultures of anaerobes are therefore much less sensitive against free oxygen than are young ones with only a small number of cells.[1]

Antagonism.—Besides co-operation, keen competition often takes place between various groups of microorganisms. The struggle for existence is no less violent, although less spectacular than among the higher organisms. Suitable food is eagerly attacked by many different species, and the more active kinds quickly outgrow the weaker ones. Rod-like bacteria are as a rule superior to cocci in this respect because of their larger working surface. Acid or ammonia producing species suppress their competitors by creating strongly acid or alkaline reactions, as in silage, milk, vinegar, or liquid manure, respectively.

Very distinctly antagonistic actions take place whenever disease producing germs try to invade higher plants and animals. Only comparatively few bacteria are able to overcome the acid reaction of plant saps, and therefore fungi are more often the cause of plant diseases. In the

[1] Burri und Kürsteiner, *Centralbl. f. Bakt.*, II. Abt., Bd. 21, 1908, p. 298.

animal, large numbers of harmless bacteria are present in mouth and intestinal tract which frequently suppress further development of single invaders of a dangerous kind. In addition, the healthy animal organism itself has, especially in its blood, various means of quickly killing and dissolving bacteria which otherwise might cause a disease.

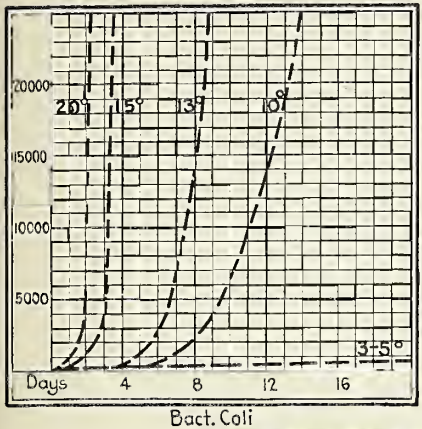
At present an antagonistie process is being extensively diseussed in seientific as well as in popular articles which is characterized by a speedy dissolution of eertain bacteria, eaused by an agent or agents not yet definitely known.[1] A French investigator, *d'Herelle,* ascribes this effeet to an "invisible" living organism, which he ealls *bacteriophage* (that is bacteria-eater), while other authors are of the opinion that soluble enzymatie substanees are to be held responsible. Undoubtedly, various eauses may lead to more or less eomplete bacteriolysis. Within the animal body the protective substances seereted by the body itself, as well as by its normal baeterial inhabitants, may exert their antagonistic action. But in addition, and especially in water and in soil, where similar baeteriophagous proeesses oeeur,[2] other organisms may be aetive which are of sueh minute size that they pass through filters which retain baeteria, and are therefore nearly or entirely invisible under the microscope. It is to be expeeted, however, that more thorough researches will reveal that these "invisible" stages of growth are connected with others elearly visible at 2000-fold magnifieation. Some observations made point in this direetion, and they indicate furthermore that this baeteriolysis may be either true autolysis (eaused by enzymatic or other substanees produeed by the baeteria themselves) or a genuine disease of the bacteria (caused by foreign organisms). Both possibilities are supported by observations,[3] whieh however need further elucidation, as does the whole problem of bacteriophagy.

Better known and of greater practical importance is the baeteriophagous aetion of *protozoa* in the intestines, in water, and in the soil. The so-ealled self-purifieation of water, as well as certain types of "soil sickness," are to a large extent the result of the elimination of great numbers of baeteria by protozoa, welcome in the first case, but disadvantageous in the latter.

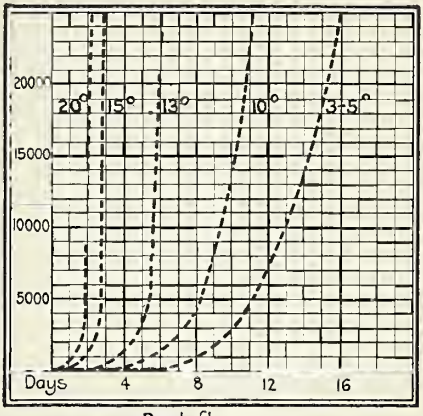
[1] Most of these papers were published in *Compt. rend. Soc. Biol.* (tomes 83–85). D'Herelle's contributions were collected in a monograph of the Pasteur Institute of Paris, entitled "Le Bactériophage" (1921), and a summary has been given by Davison in *Abstr. Bact.*, vol. 6. 1922, p. 159.

[2] J. Dumas, *Compt. rend. Soc. Biol.*, tome 83, 1920, p. 1314.

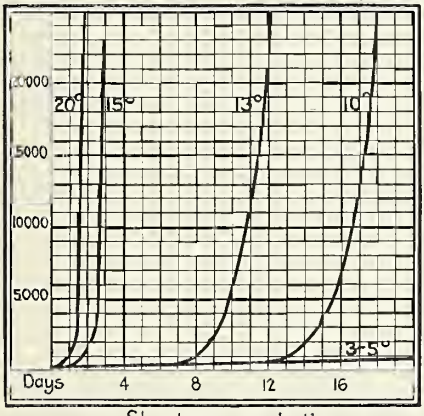
[3] E. Almquist, *Centralbl. f. Bakt.*, I. Abt., Orig. Bd. 60, p. 167; Salimbeni, *Compt, rend. Soc. Biol.*, tome 83, 1920, p. 1545; O. Bail, *Wiener klin. Wochenschr.*, Bd. 34. 1921, p. 237; Ph. Kuhn, *Berliner klin. Wochenschr.*, Bd. 58, 1921, p. 296; Pico, *Compt. rend. Soc. Biol.* tome 87, 1922, p. 836.



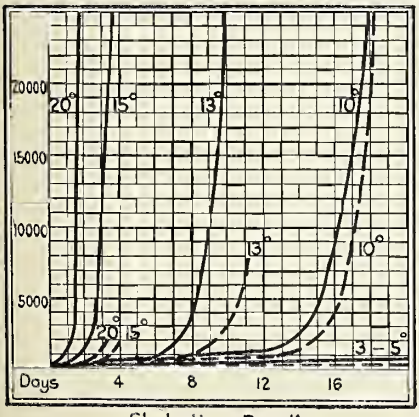
Bact. Coli



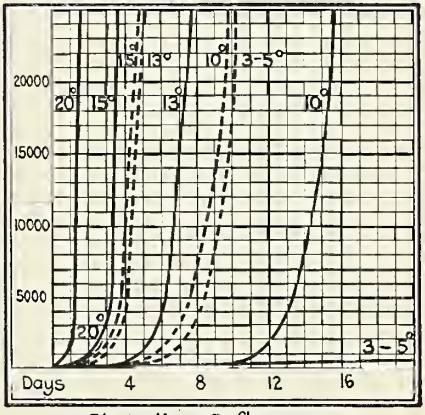
Bact. fluorescens



Streptococcus lactis



Str. lactis + B. coli



Str. lactis + B. fluorescens

FIG. 17.—Multiplication of *B. coli*, *B. fluorescens* and *Streptococcus lactis*, singly and combined, in milk at 3 to 20° C. (Numbers of bacteria are in thousands per cc.)

Experiments on Symbiosis and Antagonism.—The curves shown in Fig. 17 illustrate how such actions may be measured accurately.[1] Three of the most common milk bacteria (*B. coli, B. fluorescens,* and *Streptococcus lactis*) were grown in milk at various temperatures separately and combined. The multiplication, observed at intervals, indicates clearly that *B. coli* (frequent in the intestines and in feces) is strongly suppressed by the common lactic acid bacteria (*Streptococcus lactis*) especially at temperatures above 10° C., while on the other hand *B. fluorescens,* which digests casein and produces a slightly alkaline reaction, stimulates the growth of *Streptococcus lactis,* and is in turn itself stimulated by this symbiosis.

Chemical tests will also often prove helpful to ascertain whether symbiotic or antagonistic processes are to be taken into account. Considering the very large number of different species present and jointly active nearly everywhere, it is indeed self-evident that such investigations are quite indispensable if correct ideas of these highly complicated functions are to be secured.

4. RESISTANCE OF RESTING FORMS

The resting forms of the bacteria are microcysts, arthrospores, and endospores; those of the lower fungi, spores and conidia; and those of the protozoa, cysts (see Chapter II). They are formed in greatest numbers as soon as the environmental conditions begin to become less suitable for vegetative growth; decrease in the food supply and in the water content of the substrates exerts the most pronounced influence upon this process. If no lack of food and water occurs, as is often the case when microorganisms are grown in the laboratory (especially in milk), the inclination to produce resting forms ceases gradually and may be permanently lost. After spores and cysts are formed they are able to survive long periods in a dormant state; but as soon as food supply, reaction, moisture, and temperature are again suitable for new vegetative growth, germination will take place.

Resistance of Endospores.—Bacterial endospores are endowed with the greatest resistance against all kinds of unfavorable influences. They will hardly ever die even under the worst conditions they may encounter in nature. It needs man's action to kill them. Complete dryness and lowest temperatures are without any effect. Many seeds of higher plants show a similar behavior.[2]

[1] The countings used for constructing the curves were made by W. B. Luxwolda. *Centralbl. f. Bakt.*, II. Abt., Bd. 31, 1911, pp. 129–174.

[2] P. Becquerel, *Compt. rend. Acad. Paris,* tome 148, 1909, p. 1052; tome 150, 1910, p. 1437.

Live *steam* (of nearly 100° C.) kills bacterial spores usually after 1 to 10 minutes; some die even when temperatures of 90° to 95° C. are reached. Others, however, are much more resistant, especially those of the so-called hay and potato bacilli, normally present in large numbers upon hay, straw, and in soil. They are able to withstand the action of flowing steam for 15 to 20 hours and more, but are soon killed if the temperature of the steam is raised to 125° to 135° C. and its pressure to about 20 to 30 lbs. (1½ to 2 atmospheres). Dry air must be heated up to 170° to 180° C. before the same result is attained. Alfalfa seeds were also found to be able to survive moist heat of 100° C. for several hours, of 120° C. for 3½ hours.

If the bacterial spores are covered by protective substances their resistance may go much higher. That is especially the case when they are distributed in soil, wherein they can be killed only by very intense and prolonged heating. Occasionally truly astonishing results were obtained with certain strains of the potato bacillus (*Bac. mesentericus*) immediately after isolation from the waste lime of sugar factories. The spores survived in this case hot air of 310° to 320° C. for 30 minutes, live steam for 25 hours, boiling in 4 per cent caustic soda or in 4 per cent hydrochloric acid for 20 to 30 minutes.[1]

Resistance of Other Resting Forms.—Mold spores and conidia are generally less resistant than bacteria spores against high temperatures, but sometimes they come fairly close to them. Arthrospores and microcysts of bacteria are usually killed if the solution wherein they are suspended is heated up to 75 to 95° C. Least resistant are most of the cysts of protozoa for which the upper limit is at 60 to 70° C., while the thermal death point for the non-encysted protozoa is in the neighborhood of only 50° C.[2]

But high temperatures do not, as a rule, endanger bacterial life under natural circumstances. Lack of food and water, as well as low temperatures, are practically the only harmful physical factors in nature, harmful, however, only for vegetative cells. All resting forms are sufficiently protected to resist their influences and to safeguard the continuity of bacterial life under all circumstances.

The regenerative bodies of bacteria, although not true resting forms, may also participate in the conservation and continuation of bacterial life. Dry periods and frost do not harm them in any degree, and even against relatively high temperatures a rather strong resistance is shown occasionally. In heating tests this has repeatedly led to quite unex-

[1] Zettnow, *Centralbl. f. Bakt.*, I. Abt. Orig., Bd. 66, 1912, p. 131.

[2] A. Cunningham and F. Löhnis, "Studies on Soil Protozoa," *Centralbl. f. Bakt.*, II. Abt., Bd. 39, 1913, p. 596.

peeted results, beeause thus far the presenee of sueh bodies has usually been overlooked.[1]

5. DISTRIBUTION OF MICROORGANISMS IN NATURE

The exeeptional ability of bacteria and other mieroorganisms to make use of and to adapt themselves to widely differing and varying environmental eonditions explains the faet that they are of a truly eosmopolitan charaeter. They are more generally present on our planet than are higher plants and animals. Beeause of their minute size they are earried by wind and dust praetically everywhere, and wherever residues of higher life are to be deeomposed these highly effieient destruetive agents are present, ready to do their work. Aeeording to circumstances the *microflora* and the *microfauna,* that is the entirety of microseopic plants and animals growing at a certain loeation, differ in quality as well as in quantity, and with changing eonditions more or less far-reaching alterations take plaee in order to re-establish an equilibrium between mieroflora and mierofauna and their environment.

Germ Content of Soil.—On and in the soil originates primarily the life of all higher as well as of all lower organisms. Fertile surface soils are especially rich in rod-like, motile, and sporulating bacteria. Psyehrophilie speeies predominate in eold elimates, thermophilie in the subtropics and tropies. In soils of approximately neutral reaction baeteria are more numerous than fungi; in acid humus soils the eontrary holds true. Protozoa and lower algae are also to be found in nearly every soil; but only where the water supply is comparatively high and regular (as in greenhouses and in irrigated fields) are these two groups of organisms more or less abundant. In average field soils about 50,000 to 100,000 of the latter kinds are usually present in 1 g. soil, besides one to several hundred thousands of molds, and a few to many millions of bacteria. Not infrequently 100 million bacteria are found in 1 g. soil, where they nearly always exert the greatest aetivity. The other microorganisms taken together may represent a larger volume of living matter, but usually a less active total surfaee, because of the differenees in size discussed in Chapter I (p. 17).

One hundred million of baeteria in 1 g. of soil seems to be a surprisingly great number; in fact, however, they are not very many eompared with the spaee they occupy. One hundred millions in 1 g. of soil are equal to 100,000 in 1 mg. One gram of soil fills approximately 1 ee., and 1 mg. 1 cubic millimeter. One thousand million baeteria fill the latter spaee, if

[1] Löhnis, "Studies upon the Life Cycles of the Bacteria: Part I," *Memoirs* of the National Academy of Sciences, vol. XVI, No. 2, pp. 131, 136, and 143.

lying closely together. Accordingly, the 100,000 bacteria in 1 cubic millimeter of soil occupy only 1/10,000 of the total space available. If these bacteria were evenly distributed in the soil, and the latter could be examined in situ at 1000-fold magnification, a picture would become visible similar to that shown in Fig. 18. In reality, however, the bacteria are mostly accumulated in colonies within the soil, and the sterile stretches lying between these settlements are therefore still much wider.

But if the *weight* of these living organisms is taken into account, quite impressive figures result. One hundred million bacteria weigh approximately 1/10 mg., and 1 acre of surface soil of 30 cm. (12 in.) depth about 4 million lbs. One acre contains, therefore, about 350 lbs. of living bacteria, besides 175 to 350 lbs. of fungi, protozoa, and algae, altogether 525 to 700 lbs. per acre. The weight of cattle kept on a pasture is nearly equal to the weight of microorganisms in the soil beneath.

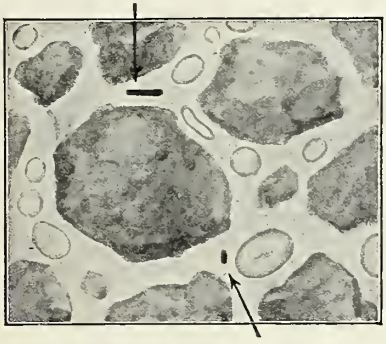


FIG. 18.—Schematic sketch of soil 1000-fold magnified, containing 100 million bacteria per g. Arrows indicate the location of the bacteria.

Generally most of the bacteria are to be found at a depth of about 10 to 15 cm. below the surface. In wet soils the upper layers are preferred; in arid soils deeper strata are also rich in bacterial life.[1] But lack of food and air tends to reduce and to exclude germ life in greater depths, although in old geological deposits, of course, traces may be found which indicate that myriads of years ago bacteria have been as active as they are to-day in disintegrating all residues left by higher organisms. Renault and other French authors[2] have made special studies upon this problem, which, however, will never be definitely solved.

[1] C. B. LIPMAN, "The Distribution and Activities of Bacteria in Soils of the Arid Region," *Univ. Calif. Pubs. in Agric. Sciences*, vol. 1, 1912, pp. 1–20.

[2] Renault's numerous contributions appeared from 1894–1900 in the *Compt. rend. Acad. Paris, Annales des sciences naturelles*, and other publications. See also BAUDOUIN, *Compt. rend. Acad. Paris*, vol. 138, 1904, p. 1001.

Germ Content of Water.—According to the amount of organic matter present in water very numerous or only very few microorganisms may be found. Pure water of deep wells is nearly sterile, but water of muddy rivers is naturally rich in all kinds of microorganisms. In the ocean more bacteria live close to the shore than on the high sea or at great depths. For drinking water 100 bacteria per c.c. was often considered a maximal number which should not be surpassed. But it goes without saying that in this as in all cases the quality of the microorganisms, not their quantity, is the decisive factor. One cholera or typhoid germ, of course, makes water highly dangerous no matter how low its total germ content may be. Mineral waters, lemonades, and other so-called soft drinks contain quite frequently large numbers of bacteria, even if they are impregnated with carbon dioxide.[1] In ponds and rivers numerous algae and protozoa, besides bacteria, are to be found; they are true water organisms and contribute to the so-called self-purification of such waters.

Germ Content of Air.—Drainage waters carry bacteria from the soil into wells and rivers; wind and dust lift them from the ground into the air. As no such possibility exists on the high sea, on glaciers, and on arctic snow fields, the air of those regions is practically free of germs. Rain, snow, and hail stones contain variable numbers of microorganisms, dependent on the amount of dust in the air, the length of time during which no rain was falling, and on the bactericidal effect of bright sunshine.[2] Accordingly, the germ content of air in large cities is generally higher than in rural districts,[3] although heavy automobile traffic over dirt roads may change this relation entirely. Usually the air in stables is highly polluted, especially if dusty hay and straw are used, and no adequate provision is made for light and ventilation. This fact is of considerable importance in the production of clean milk. Globular cells (micrococci, mold spores, and cysts of protozoa) are generally more numerous in the air than are rod-shaped cells, because the latter are less easily kept afloat by slight drafts of air.

Germ Content of Plants.—That many soil organisms are to be found on growing plants needs no explanation. But in addition to this accidental microflora there is another more specific one. Young plants raised in a germ-free environment show this clearly; the particular bacteria at-

[1] Hochstetter, *Arb. a. d. kais. Gesundh. Amte*, Bd. 2, 1887, p. 1; Thöni, *Centralbl. f. Bakt.*, II. Abt., Bd. 29, 1911, p. 616.

[2] Flemming, *Zeitschr. f. Hyg.*, Bd. 58, 1908, p. 345.

[3] P. Miquel, *Annuaire de l'Observatoire de Montsouris*, 1882; Saito, *Jour. Coll. Science*, Imper. Univ., Tokyo, vol. 23, 1908.

tached to the seed multiply rapidly and cover the whole plant with an almost continuous thin slimy layer of bacteria. The slime produced by them not only prevents their being washed off by heavy rains, but also helps to preserve a sufficient amount of moisture even during periods of drought. Besides dew, small amounts of sap excreted by the plants are available to the bacteria. Such sap contains 0.05 to 0.1 per cent organic and inorganic substances, quite enough for these modest organisms. Drying (of hay, straw, etc.) kills only part of them, while storage of fresh material, especially if fermentation takes place as in the silo, is usually accompanied by a considerable increase in number, 1000 to 2000 millions per g. being no rare occurrence in such cases. Because composition and reaction of the sap varies with the different plants, their specific microflora varies accordingly. Corn and cabbage, for instance, are rich in lactic acid bacteria, and therefore especially suited to undergo an acid fermentation in the silo and in the sauerkraut vat. The roots, too, have their special microflora, and this may contribute to the favorable or unfavorable effects noticeable with certain crops in successive plantings.

Microorganisms on and in Animals.—A young animal before birth is practically sterile, and can be kept so if taken from the mother by hysterotomy. In the normal course, however, a varied microflora soon establishes itself on the skin and within the intestinal tract. Species of bacteria adapted to higher temperature and to low oxygen tension find such conditions most favorable. In the first stomach of ruminants rapid multiplication takes place, sometimes accompanied by liberation of large amounts of gases; but later, after the acid gastric juice has been added in the fourth division, a more or less marked reduction in numbers becomes noticeable. When empty, this part of the digestive tract, as well as the small intestines, is practically sterile; in the latter case the effect of the acid is strengthened by a direct bactericidal action of the mucous lining. But a profound change occurs, and a rapid multiplication of bacteria and protozoa starts again in the last part of the intestinal tract. Again gases and offensive odors give testimony of presence and activity of numerous microorganisms. Not less than 10 to 20 per cent of the dry matter in feces is made up of living and dead bacteria. Up to 18,000 millions per g. have been found alive in solid excreta; fresh urine, on the other hand, is nearly sterile, but soon becomes strongly contaminated on the ground.

On the skin numerous microorganisms are continually deposited from the air, the litter, and the dung. They multiply in the presence of sufficient moisture; perspiration is of importance in this respect. Each gram of dirt removed by grooming contains several, frequently hundreds of

millions of bacteria, and it is obvious that by touching the flanks or the unclean udder of a cow many bacteria will be transported by the hands of the milker into the milk pail. Comparatively few microorganisms make their way through lesions of the outer skin or through the mucous membranes of the digestive tract into the inner parts of the body, which in a state of health are practically sterile. The bactericidal action of the blood, first observed by Edm. King (see p. 7), plays an important rôle in this respect. How and why pathogenic organisms may overcome this action will be discussed in Chapter VII, 6.

Germ Content of Milk and Dairy Products.—Milk produced in a healthy udder is, at first, free of germs. But contamination takes place before the milk leaves the udder. The teats with the small milk droplets left there from each milking enable the bacteria, thriving in soiled litter,

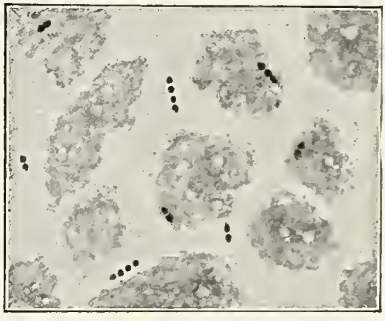


FIG. 19.—Schematic sketch of sour milk 1000-fold magnified, containing 1000 million bacteria per cc., distributed between the lighter stained flakes of casein.

to invade the udder; and though on account of the bactericidal action of the healthy body tissue most of them do not get a firm foothold, certain kinds prove sufficiently resistant to develop a specific microflora in the ducts of the udder. Every time when the milk is drawn from the udder some of these bacteria are washed out, acting as an initial contamination. But they mean little compared with the large numbers and various kinds brought into the milk with falling dirt, by contact with the milk pail and other utensils, especially if these are not scrupulously clean, that is, sterilized. As milk is rich in nutrients, rapid multiplication takes place as long as its temperature remains comparatively high (see p. 26); quick cooling is therefore of very great importance. If all possible precautionary measures are applied, the total germ content of milk can be kept at a few hundreds per cc. Ordinary market milk harbors always 1 to 10 or more millions per cc., which may be killed but not removed by pasteurization in the dairy or by boiling in the household. When milk turns acid 1000 to 2000 millions of bacteria are usually present in each cc.; such

milk, 1000-fold magnified, would present a picture like Fig. 19, which should be compared with Fig. 18. Despite the apparently very large number of bacteria present, most space is still occupied by the milk itself. Generally the same holds true with regard to butter and cheese, as was shown in Fig. 6 (p. 18). Occasionally, however, as in the whitish slimy surface layer characteristic of certain kinds of young cheese, a fairly solid layer of bacterial and fungous cells may occur, made up of approximately 500,000 million cells per g.

Germ Content of Manure.—Compared with the very large number of bacteria present in the solid excreta, those of litter and urine are almost negligible. But while in the feces practically all residues are already brought to an advanced state of decomposition, there is still much material left in litter and urine which is of value to these bacteria. Favored by the higher temperature in stables and manure piles, new multiplication starts quickly and after some days or weeks every gram of the mixture contains several thousand millions of bacteria, fungi, and protozoa.[1] If the weight of these organisms is compared with that of the manure spread on the field, it follows that in 20 tons of manure per acre approximately 450 lbs. of living matter is being added to the soil. Only part of these dung bacteria will continue to grow vigorously in the new environment, while others will die; but it becomes at once evident that this special effect of an application of animal manure must be of great importance on all soils which are not yet enriched in bacterial life by long and careful cultivation. Where hot and dry seasons tend to reduce bacterial life in the soil, regular application of well-rotted stable manure are especially to be recommended.

Adaptation to the Environment.—The cycle of matter which starts and ends in the soil is accompanied throughout its course by microorganisms which also originate in the soil and return to it. On their way they are exposed to widely varying environmental conditions, which not only cause alterations in number and species, but also lead to adaptations and to the development of new variations best suited to grow in this new surrounding. Occasionally such *local varieties* of microorganisms are of great practical importance, especially in the manufacture of butter and cheese, where they may be used as selected pure cultures. But the detrimental effects exerted by certain kinds of manuring and feeding upon the quality of dairy products are also in part known to be caused by such special varieties of microorganisms.

[1] F. Löhnis und J. H. Smith, "Die Veränderungen des Stalldüngers während der Lagerung und seine Wirkung im Boden," *Fühlings landw. Zeitg.*, Bd. 63, 1914, pp. 153-167.

Because of the close connections existing between the bacterial growths in soil, on plants, in air, water, milk, dairy products, and in manure, all fundamental studies in agricultural bacteriology must take these relations into account and must be planned accordingly. Only upon such a basis is it possible to investigate successfully the more specialized problems of dairy and soil bacteriology.

CHAPTER V

COUNTING, ISOLATING, CULTIVATING, AND TESTING BACTERIA AND RELATED MICROORGANISMS

In order to get accurate estimates of number and kinds of microorganisms present and active in soil, water, milk, manure, etc., various methods of counting, isolating, and testing bacteria, fungi, and protozoa have been developed. Most of them are not very complicated, and it is possible within a comparatively short time to acquire enough technical skill to make such investigations. A certain amount of laboratory work is indeed quite indispensable for acquiring correct ideas in regard to bacteriological problems. But the relative simplicity of bacteriological technique should certainly not create the erroneous impression that a few weeks' or months' training would be sufficient preparation for solving the most difficult problems. In addition to technical skill, a thorough knowledge of the bacteriological and agricultural literature, clear, critical thinking, exact observation, and much persistence are needed to attain really valuable results. Merely the rough outlines of bacteriological technique are given on the following pages. More detailed information may be gathered from the books mentioned on p. 11 under C and D.

Counting Bacteria and Fungi.—The very large number of microorganisms present in soil, manure, milk, etc., always makes it necessary to work with comparatively small amounts of material. But because in most cases the organisms are rather irregularly distributed and congregated in different parts of the substrate, large samples are to be taken, at first, from which after careful mixing, gradually smaller and smaller samples are prepared, if necessary, by diluting the substances with water or other liquids, wherein all living cells have been previously killed by boiling. With the smallest samples the counting is made either *directly* under the microscope, or *indirectly* by growing the microorganisms in solid or in liquid substrates. Each of these methods has its advantages and disadvantages.

Microscopic counts have become very useful for determining the germ content of milk. One one-hundredth cubic centimeter is evenly spread on a measured space of a glass slide, usually 1 cm.^{2}, dried, stained, and examined at 1000-fold magnification. The cells visible in every one of

twenty or more fields are counted, and the average number of these counts is multiplied with a factor which correlates the size of the microscopic field with the area covered by 1/100 cc. of milk and with 1 cc., respectively. If sufficient parallel tests are made, and the germ content of the milk is not too low, the figures obtained are fairly accurate; furthermore, not merely a bare number is secured, but also a certain amount of information concerning the quality of the microflora in that particular milk. The main disadvantage is that besides living bacteria a smaller or larger number of dead organisms may be visible; sometimes they are more weakly stained and can so be differentiated, but this is not always true. Such heterogeneous and insoluble materials as manure and soil are of course not suited for direct microscopic tests. It is impossible to get an unobstructed view of the bacteria; many of them are hidden behind and within the irregular particles of these substances.

Cultural tests have the advantage that they are applicable in all cases, but the disadvantage that not all organisms which are present will grow on the substrates used. In most cases solid substrates find application, and the number of colonies developing on them is used as basis for calculating the number of viable bacteria and fungi present in the material tested. The Petri dish shown in Plate III contained, for instance, the colonies which grew from 1/1,000,000 g. soil. However, the numbers obtained in this way are always below the actual number of viable germs present. Their requirements are too different to permit growth of all of them on the same substrate at the same temperature in the presence or absence of air. To make them all grow, many substrates of various composition and of different reactions, cultivation at high and low temperatures, under aerobic and anaerobic conditions would have to be used. Furthermore, many colonies are not the offspring of a single cell, but of a small or large clump of organisms. Certain groups of organisms do not grow at all on the solid substrates commonly used. In such cases *liquid* media of suitable composition are inoculated with a series of dilutions made from the original material, and it is then determined at what dilution the development ends. Heating of the substrates to 80 to 90° C. immediately after inoculation permits counting of bacterial spores and other resting forms. For enumerating fungus germs solid substrates of acid reaction are most convenient.

Counting Protozoa.—Microscopic as well as cultural tests are equally applicable to protozoa. Because most of them are larger than the bacteria, microscopic counts give fairly satisfactory results even with soil and manure. But in most cases the dilution method with solutions of various composition kept at different temperatures proves preferable. Microscopic examinations of these solutions give a good survey of the

various groups of protozoa present. Preliminary treatment of the material with hydrochloric acid makes it possible to enumerate only the encysted forms and to subtract their number from the total counts.[1]

Isolating Bacteria, Fungi, and Protozoa.—The solid and liquid substrates used for counting microorganisms permit also their being isolated in "pure culture." The first growth obtained from any given material is practically never pure, but a mixture of various kinds of organisms. If solid substrates are used the different appearances of the colonies demonstrate this fact clearly. In the case of liquid substrates microscopic tests furnish analogous evidence. But it is merely necessary to repeat the procedure with these "crude" cultures first obtained until ultimately only one type of colonies and one type of cells remain. At the time of Louis Pasteur liquid substrates alone were used, as a rule with unsatisfactory results. Solid substrates, introduced by Robert Koch, are undoubtedly superior in most cases. In fact, practically all pure culture work was done with them, and this was the basis of modern bacteriology. Nevertheless, liquid cultures may occasionally prove helpful, especially in connection with the direct isolation of single cells.

Liquid Cultures.—Liquids of different composition are the natural habitat of bacteria and protozoa in soil, manure, milk, etc. If the various food requirements are taken into account an almost unlimited number of differently composed substrates may be evolved, which are especially suitable for the growth of one or another group of microorganisms. If a complex mixture of bacterial species, as in soil, is transferred into such a solution a *natural selection* takes place; most species remain dormant and die after a while, but those adapted to the conditions offered show vigorous growth. Professor M. W. Beijerinck in Delft, Holland, has made most ingenious use of this principle, and has discovered various important groups of organisms by means of such "accumulation" experiments. Pure cultures, however, are hardly ever obtained by this method, because no single species is so highly specialized that it alone will grow under certain conditions, and even the weakest dilutions usually contain not only one, but several organisms. However, as a first step in isolating bacteria and protozoa, the use of such "elective cultures" will always be of great value.

Plate Cultures.—Gelatin (prepared from bones), agar (a partially transparent jelly made from algae and composed mostly of carbohydrates), and silica jelly are the substances most frequently used for converting liquid into solid substrates. Gelatin is rich in nitrogen and easily liquefied by many bacteria; its addition changes the general char-

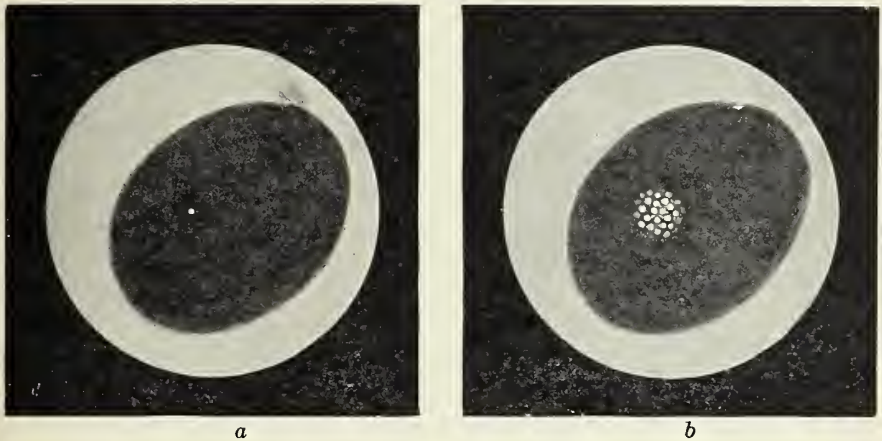
[1] D. W. Cutler, *Jour. Agric. Science*, vol. 10, 1920, p. 135.

acter of the media considerably. Agar exerts much less influence in this direction, and silica jelly practically none. According to circumstances one or the other material deserves preference. When gelatinous substrates were first used in bacteriological laboratories it was customary to spread them on simple glass plates and to call such cultures "plate cultures." Although it did not take a long time to find out that glass dishes or flasks are much more satisfactory, especially because of the better protection afforded against contamination from the air, etc., the term plate culture has been generally retained. Because the jelly prevents locomotion of motile bacteria, and the added nutrient solution permits rapid multiplication of those organisms whose food requirements are in accordance with it, colonies appear which may be the progeny of one or of several cells. Repeated platings and parallel tests lead sooner or later to really pure cultures. Thus far it has been an almost universal belief that in accordance with the monomorphistic point of view, as discussed in Chapter I, all colonies and all cells had to present a uniform appearance, if a culture was to be accepted as pure. During the last few years, however, an ever increasing number of observations has accumulated, proving beyond doubt that like the cell form, so also the appearance of the colonies of pure cultures is not as constant as was assumed. It goes without saying that this lack of constancy leads to new uncertainties and difficulties. Parallel tests and careful repetition of the platings will prove helpful in such cases, which fortunately are not very frequent. Usually fairly uniform colony growth is shown by pure cultures when originating from young cells. For thorough investigations such growth should always be selected.

Single-Cell Cultures.—Theoretically the ideal method of getting strictly pure cultures is the direct isolation of single cells and their propagation in the absence of any contamination. With comparatively large microorganisms, such as yeasts, molds, and bacteria of several micra length, single-cell cultures are not too difficult to obtain. Minute droplets of appropriately diluted liquid cultures are placed on sterile coverglasses, and after careful microscopic examination those droplets are marked which happen to contain only one single cell. Transfers are made at once or after the cell has multiplied in the droplet, which process may be watched microscopically. With bacteria of the usual minute size of ½ to 1½μ this procedure is not easily applicable. In this case the visibility of the small cells suspended in the droplet may be increased by making the dilutions in India ink [1] or similar liquids, and to place these droplets upon a layer of gelatin, where they quickly dry down. Those

[1] R. Burri, "Das Tuschepunktverfahren, 1909.

containing only one cell are marked, and either directly used for growing pure cultures (Fig. 20), or transferred to other substrates. In addition to these relatively simple methods several others have been invented, based on the use of special instruments (capillary tubes or needles) which permit the isolation of single cells directly under the microscope.[1] But these last named methods have not proved to be very satisfactory for general use. They require considerable technical skill; usually about half of the isolated cells refuse to grow; and the chances for contamination are by no means small. Therefore, as a rule, plate cultures are practically much superior to single-cell cultures, and if the results obtained are fully verified by a sufficient number of repeatedly made



a *b*

FIG. 20.—India ink droplets (×500) containing (*a*) one cell and (*b*) its progeny.

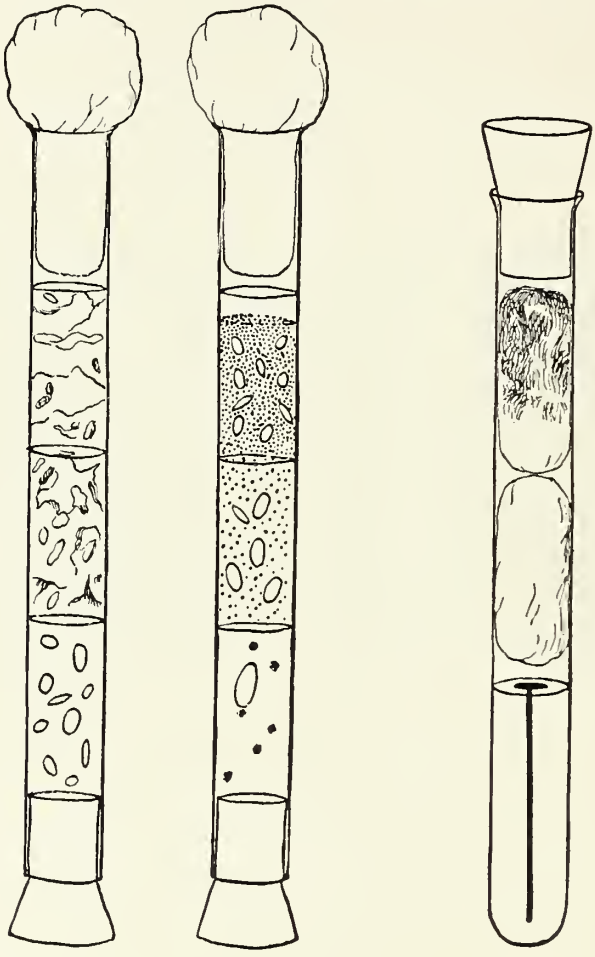
parallel tests they are equally conclusive, as all carefully made comparative tests of both methods have shown.[2]

Anaerobic Cultures.—Numerous procedures and kinds of apparatus have been devised for isolating and cultivating anaerobic bacteria. The simplest and most practicable way is undoubtedly to remove the oxygen in the cultural tubes themselves by inserting a second cotton plug which is moistened by pyrogallic acid, sodium hydrosulfite, or some other oxygen

[1] S. L. SCHOUTEN, *Zeitschr. f. Wissenschaft. Mikroskopie*, vol. 22, 1905, p. 10; vol. 24, 1907, p. 258; M. A. BARBER, *Kansas Univ., Science Bulletin*, Vol. 4, No. 1, 1907; *Jour. Infect. Diseases*, vol. 5, 1908, p. 379; vol. 8, 1911, p. 348; *Jour. Exp. Med.*, vol. 32, 1920, p. 295. HECKER, *Jour. Infect. Diseases*, vol. 19, 1916, p. 305; HORT, *Jour. Hyg*, vol. 18. 1920, p. 361.

[2] LÖHNIS, *Memoirs National Acad. Sciences*, vol. XVI, No. 2, 1921, p. 39.

absorbing liquid (Fig. 21*b*). The first isolation can be conveniently made in glass tubes, as shown in Fig. 21*a*. Three dilutions made in agar are poured above each other, and after the colonies have sufficiently developed, the rubber stopper at the lower end is removed, the agar slips



a *b*

FIG. 21.—Anaerobic cultures ($\frac{2}{3}$ nat. size). (*a*) Isolating tubes. (*b*) Stab culture.

out, and some well isolated colonies are transferred for further examination. Vigorous formation of gas makes it sometimes difficult to secure well defined colonies, as the whole column of agar is split and torn in such cases (Fig. 21*a*).

Substrates and Utensils for Bacteriological Work.—For accumulating as well as for cultivating the various microorganisms present in soil, milk, manure, etc., numerous substrates of different composition must be used. Plant decoctions, whey, or extracts made from manure and soil give frequently better results than any artificial substrates. For comparative tests, however, the latter are of great importance, and no species can be accepted as properly described which has not been thoroughly tested at least on the following media: Beef agar without and with

FIG. 22.—Arnold Sterilizer ($\frac{1}{15}$ nat. size).

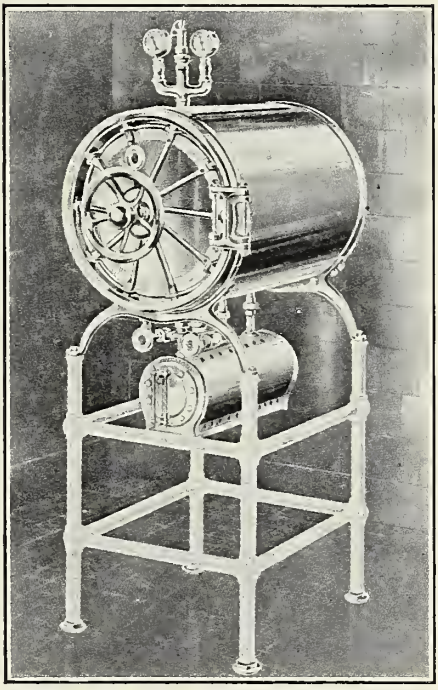


FIG. 23.—Autoclave ($\frac{1}{20}$ nat. size).

0.5 per cent glucose, beef gelatine, beef broth, milk, and potato. The beef substrates are all made from broth, prepared either from fresh meat, from meat extract, or from dried media furnished by several firms. Details to be observed in the preparation of these and of other media are to be found in the laboratory manuals mentioned under D on p. 11.

After being prepared the substrates are filled into test tubes, which are closed with cotton stoppers destined to exclude all outside germs. All bacteria and molds present within the media are killed by thorough heating. This is done either in live steam in a sterilizer of cylindrical or

eubical shape (Fig. 22), or under pressure in a so-ealled autoclave (Fig. 23). Heating in the autoelave permits a more rapid and thorough sterilization, but not all media can stand this most severe treatment. For these, live steam is to be used; but on aceount of the great resistance of some of the baeterial spores, repeated heatings, the so-called fractional sterilization, is required in this case. After each heating all or part of the surviving spores will germinate, and their progeny will then be killed by the next heating, usually on the following day. If the steaming is repeated at five suceessive days, as a rule the media will be sterile, although substrates exceptionally rich in highly resistant spores may still contain contaminating organisms.

Empty glass vessels, Petri dishes, ete., are placed in a hot air

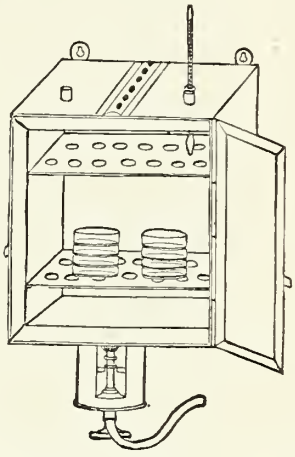


Fig. 24.—Hot air sterilizer ($\frac{1}{15}$ nat. size).

sterilizer (Fig. 24) and exposed to temperatures of about 165° C. Transfers of bacterial growth are made with platinum needles and loops, which immediately before and after use are made red hot in an open flame.

Testing Pure Cultures.—Because of the omnipresence of mieroorganisms it is of preeminent importanee carefully to protect pure eultures onee obtained against all contaminations. Sterilized containers, sterilized media, and sterilized utensils are absolutely necessary. Parallel tests are always to be recommended. The development of pure eultures upon the various substrates mentioned above is usually very eharaeteristic. Plate V. illustrates this faet with regard to *B. coli,* an intestinal species and therefore common in manure and in unclean milk, and *B. prodigiosum,* characterized by its red pigment best noticeable on agar and on potato. Gas formation by *B. coli* is visible in the glucose agar

stab as well as in milk; but only a trace of red is produced on top of the milk culture of *Bact. prodigiosum.*

Frequently such cultures are kept for only a week, and many species descriptions have been based upon such short termed and totally insufficient observations. All cultures should be tested at frequent intervals during at least one month and preferably longer. Repeated tests are necessary in order to collect information upon constancy or variability of the strains studied. Professor J. G. Adami[1] once urged that everybody who publishes a description of a new bacterial species, should furnish one year later a second description based on renewed studies. Perhaps it would be still better to extend all such investigations for a whole year or longer; in this way the careless "species" making would be materially reduced.

Microscopic Studies.—Cultural tests should always be accompanied by microscopical studies, which are not to be restricted to one or a few

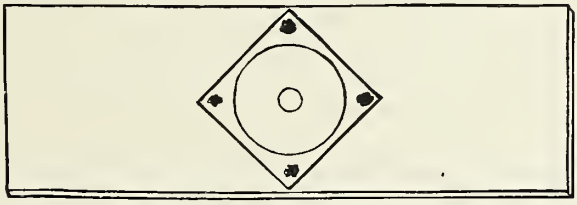


FIG. 25.—Hanging drop preparation (nat. size).

days, as is frequently done, but they too are to be continued as long as changes become visible in order to get a complete knowledge of the morphological characters. These tests are to be made both with living unstained material suspended in water or nutrient solution, and with dried and stained preparations; the latter are as a rule more satisfactory, especially with bacteria. Plate I shows the different appearance of the organisms when treated in these various manners.

For observations of living bacteria so-called *hanging drop preparations* are most suitable. A small droplet containing the bacteria is placed on a coverglass, and this is fastened upon a hollow slide so that the drop comes in the center, not touching the sides nor the bottom of the depression (Fig. 25). Especially at the edge of the drop the cells are clearly visible, and their motility or immotility can be ascertained. Some workers prefer to have the liquid in a thin flat layer; this can be accomplished by covering the drop with a second coverglass of smaller diameter, or by placing a small piece of agar against it (so-called hanging agar block preparation).

[1] *Publ. Health Papers and Rep.*, Amer. Publ. Health Assn., vol. 20, 1894, p. 415.

Stained preparates are usually made in the following manner. The bacteria are spread evenly on a coverglass or upon an ordinary glass slide, and allowed to become dry. They are then fixed by quickly heating in the open flame, or by applying special fixatives (alcohol, osmic acid, etc.) so that they stick firmly to the glass, and stained by pouring upon the area covered by the bacteria an aqueous solution of some anilin dye, such as methylene blue, fuchsin, Victoria blue, gentian violet, etc. After a few seconds or minutes the staining solution is poured off, the slide or the coverglass is thoroughly rinsed with water, and after being dried it is ready for inspection. For fastening the coverglass upon a slide Canada balsam is mostly used, which becomes quite hard after a few days. Such mounts, if properly made, keep indefinitely. Very convenient, although not always applicable, is the method of mixing the bacteria with India ink before spreading them upon the coverglass or the glass slide. Such smears need no fixing or other treatment, give very clear pictures, provided that slime or other disturbing elements are absent, and show details within the cells which in stained preparates can be brought out only by special, more complicated procedures.

Up to about 500-fold magnification the ordinary dry lenses are quite satisfactory. For 1000-fold magnification, however, which is usually necessary in bacteriological work, some special appliances are generally used which were invented by Professor Ernst Abbe at the Zeiss works in Jena, just at the time when Robert Koch began his important investigations. The first one is a *condenser,* which concentrates a large amount of light upon a very small part of the preparation, and the second one is a so-called *immersion lens,* that is a special type of objective which is to be immersed into a drop of cedar oil, placed upon the coverglass or directly upon the dried smear. This combination tends to direct as much light as possible into the microscope and into the eye of the observer, and assures therefore very clear and sharp pictures, which otherwise could not be obtained.

Additional Tests.—Special tests concerning the behavior of pure cultures at high and at low temperatures, toward various sources of carbon and nitrogen, etc., will often be added advantageously to the ordinary cultural and microscopical tests. A descriptive chart, worked out and revised from time to time by a Committee of the Society of American Bacteriologists, furnishes valuable details in this direction, but again it must be strongly emphasized that such experiments are of real value only if they are extended over long periods, checked by parallel tests, and repeated several times.

Furthermore, symbiotic and antagonistic effects deserve careful consideration. Experiments with mixed cultures under natural conditions

are necessary to complete and to confirm pure culture studies. In the same manner as the medical bacteriologist combines his microscopical and cultural investigations with animal tests, so it is necessary that the agricultural bacteriologist carry his work from the laboratory to the dairy, to the greenhouse, and to the field, in order to get results of really scientific as well as of practical value.

CHAPTER VI

STERILIZATION, PASTEURIZATION, ANTISEPSIS, AND ASEPSIS

At present, numerous methods are available for eliminating or suppressing unwelcome or harmful microorganisms. Several of them have been used since ancient times, but only since modern bacteriology has shed light upon life and activities of bacteria has a more rational and successful fight against these minute enemies of mankind become possible. Thorough knowledge of their properties, especially of their behavior toward external influences, has served as a basis for developing the modern methods of sanitation. The vast majority of microorganisms, however, are not harmful, but useful to mankind, and it is therefore important to select in each case the proper procedure in order to reach the desired effect without seriously disturbing the action of useful organisms.

Effects of Various Methods.—With regard to the more or less thorough elimination or suppression of unwelcome microorganisms the methods available are to be classed as follows:

(*a*) *Sterilization* or *disinfection,* aiming at the complete destruction of all organisms present. The term disinfection is used as a rule when special attention is paid to the killing of pathogenic (infective) bacteria, while the word sterilization indicates destruction of all microorganisms present. But this goal is not always reached; the high resistance of bacterial spores makes sterilization often incomplete.

(*b*) *Pasteurization* and *antisepsis,* aiming at the destruction of the majority of organisms present, especially of those in the vegetative state. Again the second term is used mostly in medical bacteriology, while pasteurization is of more general application.[1]

[1] The term antisepsis is derived from the Greek words ἀντί (anti-) = against, and σῆψις (sepsis) = putrefaction, decay. The term pasteurization was introduced to honor the memory of Louis Pasteur. The method itself was known before Pasteur's time; about fifty years earlier it was used by Appert, by the great French chemist Gay-Lussac, and by others.

(*c*) *Asepsis,* aiming at the complete exclusion of microorganisms and prevention of development of those present. The meaning of the term is that no putrefaction takes place. In the treatment of wounds especially, and also in other cases, asepsis is as obviously preferable to antisepsis, as is prevention to cure.

The treatment directed against microorganisms may be *physical* or *chemical* or both combined. Always the relatively most efficient procedure must be selected to suit the individual case; there is, of course, no single treatment best suited to all cases. The question of greatest economy requires careful consideration in this connection.

Most radical and most efficient is the direct application of the open flame. In earlier times this extreme measure had often to be relied upon when serious epidemics swept the countries. Houses, goods, and corpses had to be destroyed by fire in order to check the disease. Even to-day such a procedure may occasionally become necessary, and the cremation of bodies appears from this point of view distinctly superior to their interment even in normal times.

With all other less thorough methods of sterilization and pasteurization real sterility is not easily attained. But even if all germs are killed, their bodies, and the harmful metabolic products which they may have produced, are still there, and the very wide-spread assumption that milk, for instance, could be "freed" from all detrimental bacteria by thorough heating, is not correct. The germs are merely killed, but not removed, and their products may still be active. Perfectly clean milk, aseptically handled, is much more preferable; its high cost, however, prevents its general use.

Physical Treatment.—That low temperatures merely stop further growth and action of bacteria, but exert otherwise very little effect upon them, has been emphasized in Chapter IV, 2. *High temperatures,* on the other hand, are of fairly satisfactory effect, especially in the presence of sufficient moisture. Moist air or *steam* is always much more effective than dry air of the same temperature. Most vegetative cells are killed by moist heat at temperatures ranging from 50° to 70° C., more slowly, of course, at the lower, more rapidly at the higher temperatures. In Swiss cheese factories temperatures of 50° to 55° C. are applied to milk and curd in order to destroy yeasts and other organisms which might prove harmful if they were allowed to grow. In haystacks and manure piles partial sterilization by spontaneous heating is quite common. Holding milk for 20 to 30 minutes at 63° C. has proved to be the best method for pasteurizing market milk, because about 99 per cent

of the undesirable organisms are killed, while flavor and other qualities of the milk are not much impaired. With cream (for butter making), as well as with skim milk, a short time exposure at 85° to 90° C. is preferable. But complete sterilization of milk is an extremely difficult task except in those cases where resistant spores are entirely absent. More than 104° C. can not be applied without making the milk unpalatable, and despite repeated heatings some spores may survive, which will later germinate and cause spoilage of such an incompletely sterilized product. Complete sterilization is assured only if the temperature in the autoclave is kept for one hour at 116° to 120° C., as is done in preserving meat and vegetables. Nevertheless, some spores may survive even this severe treatment, although more frequently spoilage occurs afterwards because imperfect containers permit new contaminations.

In order to keep the germ content of milk low from the start, the use of sterilized utensils is of greatest importance. In the production of certified milk this point requires continual attention. In America steam treatment is still often applied in such cases, but investigations made in Europe about 20 years ago have definitely shown that *hot air* treatment is much preferable. The moisture remaining in steamed vessels facilitates new contaminations and new growth of bacteria, which possibilities are entirely eliminated in the other case. Glass containers withstand 165° C. very well, which temperature is usually applied in bacteriological laboratories, but metal utensils are better kept for a longer time (about 5 to 10 minutes) at a somewhat lower temperature (130° to 140° C.).

Drying at low temperatures kills only comparatively few of the vegetative cells, but artificial drying *at high temperatures* is very efficient. Dried milk, dried potatoes, etc. are practically sterile after treatment, but contamination soon sets in again.

Several investigators have tried to pasteurize milk by exposing it to *ultra-violet rays;* the results were quite unsatisfactory. But an analogous treatment is used effectively for water purification. Several French cities have adopted it for their water supplies.[1]

The mechanical elimination of bacteria by *filtration* is widely used for reducing the germ content of water. Natural filtration takes place in every soil; therefore, water from deep wells is practically sterile. Where surface water must be used it is sent through special filter beds constructed of sand and gravel (Fig. 26). Most of the bacteria are retained by the layer of mud which accumulates in the uppermost part of the sand. The speed with which the water passes the filter must be carefully regulated, and the efficiency of the filter must be regularly controlled by bacteriological tests.

[1] M. von Recklinghausen, *Journ. Franklin Inst.*, vol. 178, 1914, p. 681.

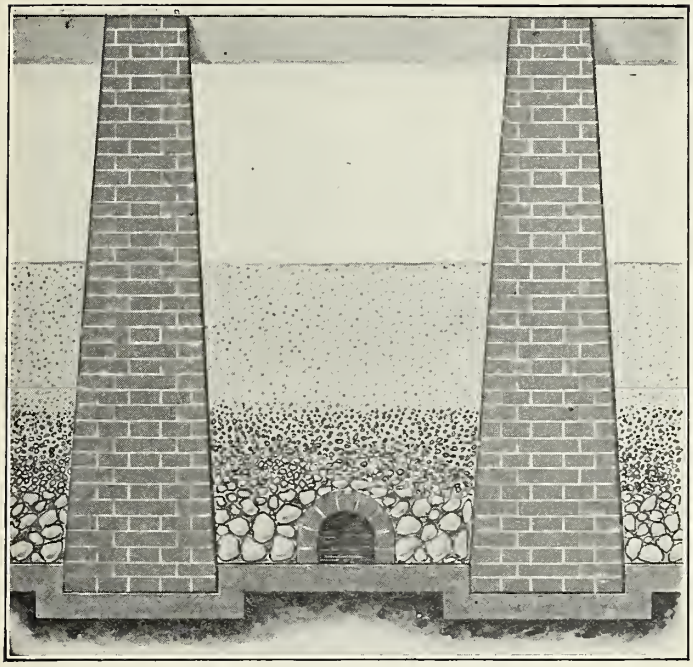


FIG. 26.—Filter beds ($\frac{1}{40}$ nat. size)

FIG. 27.—Chamberland. Bougies ($\frac{1}{6}$ nat. size)

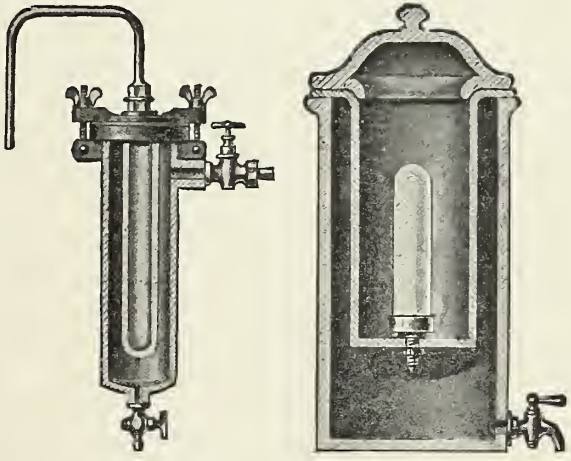


FIG. 28.—Berkefeld filters ($\frac{1}{6}$ nat. size) for running and for stored water.

Complete elimination of bacteria and other microorganisms from water is possible by the use of special bacteria filters, such as the *Chamberland filter,* shown in Fig. 27, or the *Berkefeld filter,* as seen in Fig. 28. The filtering is done through so-called bougies, porous cylinders made from porcelain, asbestos, infusorial earth (kieselguhr), cellulose, or similar materials, closed at one end, with pores of less than 0.2μ diameter. In bacteriological laboratories such filters are used for sterilizing substrates which do not stand heating, but the results are not always satisfactory. If the solutions which are to be sterilized contain colloidal substances a smaller or larger part of these will be retained by the filter and will clog it eventually. Another point is that always more and more organisms are found to be able to pass such filters, even if these are absolutely perfect, that is, free from fine cracks and holes large enough to let bacteria of average size pass through. Especially among the disease producing organisms such filterable forms are well known, as for instance with rabies, foot and mouth disease, hog cholera, small-pox, trachoma, infantile paralysis, typhus fever, Rocky Mountain spotted fever, etc. But in addition to these, probably numerous non-pathogenic germs are filterable, especially many of the gonidia of the smaller types of bacteria. Furthermore, even those bacteria which because of their size can not pass the pores, have often shown themselves capable of growing as very thin threads through the filter, if these are continually used for a long time. This possibility is to be kept in mind if such filters are permanently used in the household in the belief that they assure a perfectly safe water supply, which they do not.

Air can be freed from bacteria and mold spores by drawing it through sufficiently thick layers of *cotton,* as is done, for instance, in certain types of milking machines. It is frequently assumed that the milk itself could by freed of its bacteria by sending it through a cotton filter. But comparatively few of them are removed, namely those clinging to the dirt which remains on the cotton. All others pass through the filter, because they are smaller than the fat globules of the milk, whose diameters vary usually between 4 and 10μ.

Simple mechanical removal of bacteria by *scouring* and *scrubbing* is but partly successful, especially in those cases where the bacteria are resting upon a perfectly smooth surface. But any irregularities of the surface, even if they are hardly visible to the naked eye, may afford complete protection to the bacteria. Ordinary cleanliness is therefore by no means identical with bacteriological cleanliness, which to attain requires a more thorough treatment.

Chemical Treatment.—Many "antiseptic" or "disinfectant" substance are known at present, and new ones are recommended nearly

every day. They are very helpful in the fight against diseases, but their use for preserving food is rightly restricted. The "pure food laws" properly forbid their use or demand that their presence be clearly mentioned.

Whether a chemical substance is harmful or not depends mostly on its *concentration.* Distinctly poisonous compounds become harmless to bacteria if present in very small quantities, or they may even exert a stimulating effect. On the other hand, substances which normally act as nutrients, may become harmful if consumed in too large quantities. Furthermore, the different degrees of *sensitiveness* of the various microorganisms, and especially the generally high *resistance* of their resting forms must be taken into account, in order to understand correctly why the results obtained by chemical as well as by physical treatments may vary widely according to circumstances. As was pointed out before, enzymes are usually more resistant than is the living cell, and metabolic processes may therefore still proceed after all living cells have been killed by chemical treatment; in other words, bacterial propagation is more easily suppressed than is bacterial activity.

Among the various substances available in the household for the "chemical warfare" against microorganisms, *acids* are often used advantageously because the majority of bacteria are very sensitive against a distinctly acid reaction. The weakest acid, *carbon dioxide,* exerts only a slightly retarding effect, as was pointed out on p. 62. Sauerkraut, sour pickles, and silage contain usually 1 to 2 per cent *lactic, acetic,* and other *organic acids,* which suffice to stop practically all bacterial development; but molds may cause spoilage unless their growth is suppressed by the absence of air. 2 to 3 per cent *mineral acids,* like sulfuric and hydrochloric acids, are sometimes used for treating manure from diseased animals, or for sterilizing wood work, rubber parts, etc. The antiseptic effect of different acids is not dependent on their hydrogen ion concentration, but apparently on their ability to penetrate the cell wall. Burri noticed, for instance, the following relative efficiencies of various acids towards several milk bacteria:[1]

Hydrochloric acid	100	Acetic acid	100
Nitric acid	100	Propionic acid	100
Sulfuric acid	80	Lactic acid	100
Phosphoric acid	60	Citric acid	40
Formic acid	100	Tartaric acid	20

Basic substances must be applied usually in higher concentrations. *Slaked lime* proves very useful in stables and dairies. In the soil, too, it

[1] Burri, *Landw. Jahrb. d. Schweiz,* Bd. 26, 1912, p. 475.

can be used for at least partial sterilization, if applied in large quantities. Still more efficient than lime alone is a mixture of equal parts of milk of lime and of a 20 per cent solution of caustic soda. 2 to 5 per cent *sodium carbonate* is economical and very active if applied at temperatures of 60 to 80° C. 5 to 10 per cent caustic potash mixed with 10 per cent sodium hypochlorite makes a very strong disinfectant, known as *antiformin*. Unfortunately, the tubercle bacilli are resistant against all these substances, excepting only hot sodium carbonate solution. Antiformin is used to separate tubercle bacilli from other species in the examination of sputum and other pathological material.

Among the *mineral salts* mercuric chlorid, commonly known as *corrosive sublimate*, is usually considered to be one of the strongest poisons. 0.1 per cent often suffices to kill all bacteria. But wherever protein substances are present, an insoluble compound is formed, and the effect is greatly reduced. *Copper sulfate*, widely used for controlling plant diseases caused by fungi, may also be advantageously applied to check the growth of algae and bacteria in water reservoirs. If the water is not rich in carbon dioxide, concentrations as low as 1 part per million have proved effective.[1] *Chloride of lime* is also very helpful for safeguarding the water supply (in most cases 1 to 3 parts per million will suffice), for reducing the germ content of dairy utensils, and as a disinfectant of manure and sewage. *Ammonium fluoride* (0.5 per cent) is especially suitable for the sterilization of rubber tubes. *Potassium permanganate* and *potassium bichromate* are also efficient in relatively low concentrations (½ to 1 per cent). *Sodium chloride*, on the other hand, becomes active only in very high concentrations, and the effect remains incomplete. Even 25 per cent salt does not exclude all growth of bacteria and yeasts.[2]

Besides chloride of lime, *ozone* is widely used for water sterilization. Many European cities, as Paris, Nice, Florence, have adopted this method. Ozonization of milk has also been tried, but without satisfactory results. Somewhat more favorable was the application of *peroxide of hydrogen*. In Denmark this treatment was fairly extensively used when milk was shipped for long distances, and it is of indisputable value for preventing losses in times of milk shortage. It retards bacterial growth distinctly and is quite harmless, because it splits up into water and oxygen, but as long as small amounts are still present in unchanged state the taste of the milk is inferior. Peroxide of hydrogen is of greatest value for the antiseptic treatment of wounds.

[1] U. S. Dept. Agr., *Bur. Plant Industry Bull.* 64, 76, 100.

[2] Wehmer, *Centralbl. f. Bakt.*, II. Abt., vol. 3, 1897, p. 209; F. Lewandowsky, *Archiv f. Hyg.*, vol. 49, 1904, p. 47.

Best known among the *organic disinfectants* and widely used are *phenol* (*carbolic acid*) and related compounds. But in order to get a full effect a 5 per cent solution must be applied, preferably at about 40° C. This makes such disinfection rather expensive, and the strong odor of these substances is liable to act unfavorably upon milk and other food it may reach. Under various trade names special preparations are sold which do not smell quite as strong as does carbolic acid, but their price is usually too high. Very efficient and economical is *formaldehyde*, in ½ to 1 per cent concentration useful for dairy utensils, rubber tubes, etc. In combination with caustic lime or permanganate it is of special value for the fumigation of rooms (5 g. formaldehyde per cbm. space). After 3 to 4 hours the remaining formaldehyde is removed by adding ammonia (3 g. per 5 g. formaldehyde). High humidity of the air and a high temperature are essential for securing the best possible results. *Benzoic* and *salicylic* acids are not infrequently used in the household for protecting food against spoilage. Three-tenths per cent is an efficient and still fairly harmless amount; nevertheless, complete sterilization by heat is preferable to the use of any chemical substances.

Combined Treatment.—For practical purposes the simultaneous application of several kinds of treatment is, of course, often feasible and desirable. The action of chemical substances can be considerably increased by a change in reaction, as well as by an increase in temperature. Ordinary wash suds give practically complete sterilization if they are used at or near the boiling point. Heat, high concentration, and acid reaction combine their effects in the sterilization of fruit preserves. High pressure, which otherwise is not very reliable, has proved to be valuable for the treatment of acid fruit juices;[1] the surviving spores can not germinate in the acid medium. Electrical pasteurization of milk, which was tried in Liverpool, England, and in American army camps,[2] acts by raising the temperature to about 70° C. and perhaps by simultaneously starting some electrochemical reactions within the cells. Meat preserved by smoke is at the same time dried and exposed to formaldehyde and other antiseptic substances in the smoke. Salting of meat and fish acts by increasing the osmotic pressure, as well as by the chemical effect of sodium chloride and other compounds present in ordinary salt. Many other combinations of physical and chemical action are possible; they are the most practicable means of preventing detrimental bacterial action.

[1] Hite, Giddings, and Weakley, W. Va. Exp. Sta. *Bull.* 146, 1914.

[2] A. K. Anderson and R. Finkelstein, *Jour. Dairy Science*, vol. 2, 1919, p. 374.

CHAPTER VII

ACTIVITIES OF BACTERIA AND RELATED MICROORGANISMS

The continuity of life on earth is dependent upon the uninterrupted progression of the cycle of matter, as was discussed on p. 2. There is an equilibrium between the constructive work done mostly by higher organisms, and the destructive work done mostly by microorganisms; the latter have been properly called the "mediators between death and life" of higher plants and animals. Under this aspect the action of pathogenic bacteria represents a transgression of the proper domain of bacterial life, because it accelerates death and hastens decomposition. But the activities of bacteria and related microorganisms are by no means always destructive, as those of the higher organisms are not always constructive. Large amounts of the organic compounds formed by the latter are again destroyed in the process of respiration; certain groups of bacteria, on the other hand, are doing eminently constructive work, as for instance the nitrogen-fixing bacteria in the root nodules of leguminous plants.

Efficiency and Virulence.—As was emphasized in the preceding chapters, it is the minute size, the simple but effective structure of the bacterial cells, and their capability of very rapid multiplication under very different environmental conditions which cause their stupendous efficiency. One or a few bacteria are practically without significance; but if suitable circumstances favor their multiplication and activity, very soon conspicuous changes will result. H. W. Conn has drawn a very appropriate comparison between bacteria and snowflakes; singly of nearly no weight and very short-lived, they are both able to become very powerful if present in very large masses. Avalanches destroy men and their homes, bacterial epidemics may depopulate human settlements; and enormous economic losses are caused year after year by microorganisms liberating nitrogen. Fortunately, however, the useful activities of bacteria, as a rule, exceed their detrimental effects; and the better their properties are known the more advantageous use can be made of them.

Pathogenic bacteria act in most cases by the toxic substances they produce; accordingly, it has become customary to speak of their *virulence* in order to refer to their poisonous properties.[1] Unfortunately, the same

[1] Derived from the Latin word virus (plur. vira) = poison.

word is not infrequently used for non-pathogenic bacteria, too. Several authors have written upon the "virulence" of lactic acid bacteria, of nitrogen fixing, and of other useful microorganisms, where they meant, of course, their *efficiency,* as there is no poison produced in these cases. Obviously, the words efficient and virulent can be used synonymously only for pathogenic, not for other organisms. Expressions like "virulence of fermentation," which also may be found occasionally in the literature, are, of course, quite incorrect and should be strictly avoided.

Relativity of Action.—Like the functions of all other living organisms so is the action performed by microorganisms always dependent on their efficiency, as well as on the modifying influences of outside conditions. Good seed will usually produce better crops than can be expected from poor seed, but circumstances may arise which will completely change these relations. Good seed is not of much use in a badly tilled poor soil, and the best milk cow can not display her efficiency if proper feed and care are lacking. The situation is exactly the same with the lower organisms. Pure cultures of the highest efficiency could be selected and cultivated in the laboratory, but they would not exert any appreciable effect under unsuitable conditions in the dairy or in the soil. Therefore, proper environmental conditions are no less important than is the efficiency of the organisms concerned.

But outside conditions differ and vary, as does the efficiency of the bacteria. Numerous factors exert their influences, and the final result is determined by the relations existing between them. The great variability of bacterial cells and the ability of most microorganisms to adapt themselves to very different environmental conditions lead to changes in bacterial action unknown among the higher organisms. Such characteristic functions as the production of lactic acid, of slime, of ammonia, and the fixation, or the liberation of nitrogen may not only show wide variations, but they may cease entirely; therefore, in the laboratory great attention must be paid to keep the cultures at their highest efficiency.

Physical and Chemical Actions.—Chemical as well as physical actions take part in the transformation of matter performed by the lower as well as by the higher organisms. From a practical standpoint the metabolism of carbonaceous and nitrogenous substances is undoubtedly of greatest importance. The chemical changes occurring in the silo, in ripening cream and cheese, in rotting manure, and in the soil will be discussed on the following pages. But in connection with these transformations various physical effects of bacterial activity are to be observed, and sometimes these physical functions appear of special interest. This holds true particularly with regard to the production of color, of

light, and of heat by various microorganisms, which will be briefly considered first.

1. PRODUCTION OF COLOR, OF LIGHT, AND OF HEAT

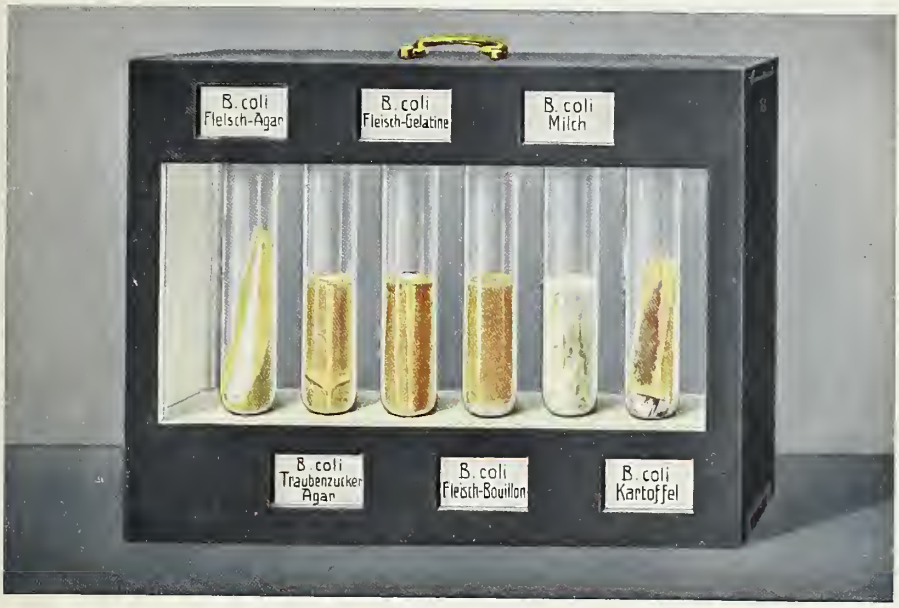
In regard to the physical functions, again many parallelisms are noticeable between the behavior of higher and of lower organisms. Pigmentation is very common among lower and higher plants and animals; numerous light producing organisms, foremost fishes, have been found especially in the deep sea; fireflies and other phosphorescent insects are well known; and the production of heat is noticeable wherever intensive respiration takes place.

Pigment Formation.—The majority of microorganisms do not display any pronounced pigmentation. Most cultures of bacteria and yeasts appear as whitish or grayish, soft, slimy or dry layers covering the substrates, without distinct character if examined with the naked eye. In Fig. 1, Plate V, this whitish growth of *Bact. coli* is clearly visible; on potato only is a brown pigment in evidence. Inspection of a yeast cake will demonstrate the analogous appearance of a colorless fungus. Molds, too, grow mostly without color, at least as long as no spores are formed. Their white network of threads is quite common on sour cream, stale bread, old leather, etc. When growing in liquids, grayish loose flakes are formed.

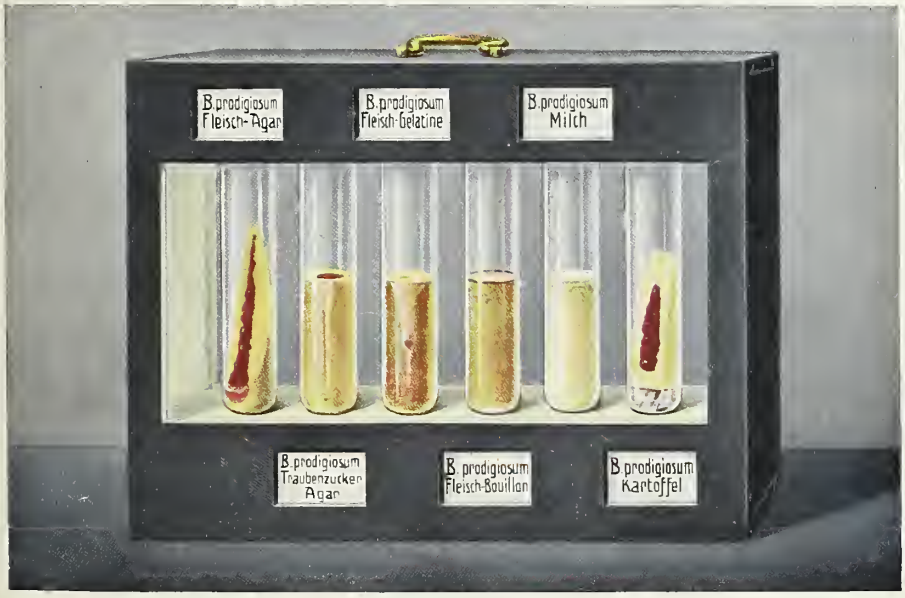
If colored growth appears, the pigment either remains within the cells which show the coloration, or, less frequently, it leaves the cells as an excretion which diffuses into the substrate, as may be seen around some of the colonies visible in Fig. 1, Plate III. On the other hand, some molds as well as bacteria have the tendency to extract certain coloring substances, for instance Congo red, from the substrate and to accumulate it in their cells. This ability is sometimes of diagnostic value, and it also explains why with several molds strains of different coloration occur, which in such cases is merely accidental.[1]

Next to white or gray, a *yellow* pigmentation is most frequent among the bacteria as among flowers. All hues from pale lemon color to a deep rich orange may be seen (Plates III and VI). *Pink* and *red* tints come next in frequency. A pink yeast and *Bact. prodigiosum* are shown on Plates III and V. A bright red coloration of the substrate may be due to the growth of various molds or of *Bact. erythrogenes,* whose yellow pigmented cells present a striking contrast to their red surrounding (Plate VI). Purple colored bacteria occur sometimes in very large numbers in the water of ponds and ditches, where they may participate in

[1] Marzinowsky, *Archiv f. Hyg.*, vol. 73, 1912, p. 191.



1. Cultures of Bacterium coli
$^1/_3$ nat. size



2. Cultures of Bacterium prodigiosum
$^1/_3$ nat. size

the oxidation of hydrogen sulfide. A soluble fluorescent substance, presenting a *green* or *bluish green* color in reflected light, is produced by *Bact. fluorescens,* one of the most common organisms in water, milk, manure, and soil, and by *Bact. pyocyaneum,* a species connected with pus formation in wounds and sometimes in the udder. *Bact. fluorescens* is very active in splitting fats and in producing ammonia, and therefore of special interest. A yellowish-green, bluish-green, or grayish-green pigmentation is frequently noticeable with the conidia of various molds. *Brown* and *black* colors are also not rare with lower fungi as well as with bacteria. Brown or black varieties of *Bact. fluorescens* are often met with in stable manure. Some strains of the hay and potato bacilli (*Bac. subtilis* and *mesentericus*) are also able to produce a black pigment, which is very characteristic of one of the most important nitrogen fixing species, *Azotobacter chroococcum.* Another interesting group of soil organisms, usually named *Actinomyces chromogenes,* is characterized by a soluble brown pigment, visible around one colony in Fig. 1, Plate III. *Blue* and *violet* colors are also not absent among bacteria and fungi. Best known of these is one species, called *Bact. syncyaneum* or *B. cyanogenes,* which produces in slightly acid milk an intensive sky-blue color (Plate VI); occasionally black strains occur with this species, too.

Practical Importance of Pigment Bacteria.—Before the milk separators were invented, and the milk had to be kept several days before the cream could be taken off, blue, yellow, red, or green discolorations of the milk were rather frequent. Sometimes they proved extremely troublesome, as for instance in the case which ultimately led to the discovery of *B. cyanogenes.* On this particular farm all milk turned blue during eleven years, and the owner went bankrupt before the cause was discovered and remedies found. Red milk was and is also not rare. If it is red from the start, admixture of blood from a diseased udder is the cause; but if the color appears later, almost invariably *Bact. erythrogenes* is responsible. Repeatedly *Bact. prodigiosum* has been blamed, but, as was pointed out above, this species produces only a faint pink color in the cream of its milk cultures, which fact is of no practical consequence. Yellow spots on cream and a greenish discoloration of milk are often to be observed if milk of low germ content is kept for a long time at low temperatures (2 to 5° C.).

Bacterium prodigiosum has received its name the "wonder worker"[1] because of its connection with the sudden appearance of red spots on bread, meat, and especially on the consecrated wafers kept in the dark,

[1] The Latin word prodigium means wonder.

damp, medieval churches of Europe. Sometimes these "bleeding hosts" were merely aeeepted as wonders and served to stimulate religious zeal, but in other eases quite innocent people, especially Jews, were held responsible for having caused these "wounds on the body of our Lord"; and, as far as records have been kept, about 10,000 men and women have lost their lives due to fanatic persecution by exeited ignorant mobs.

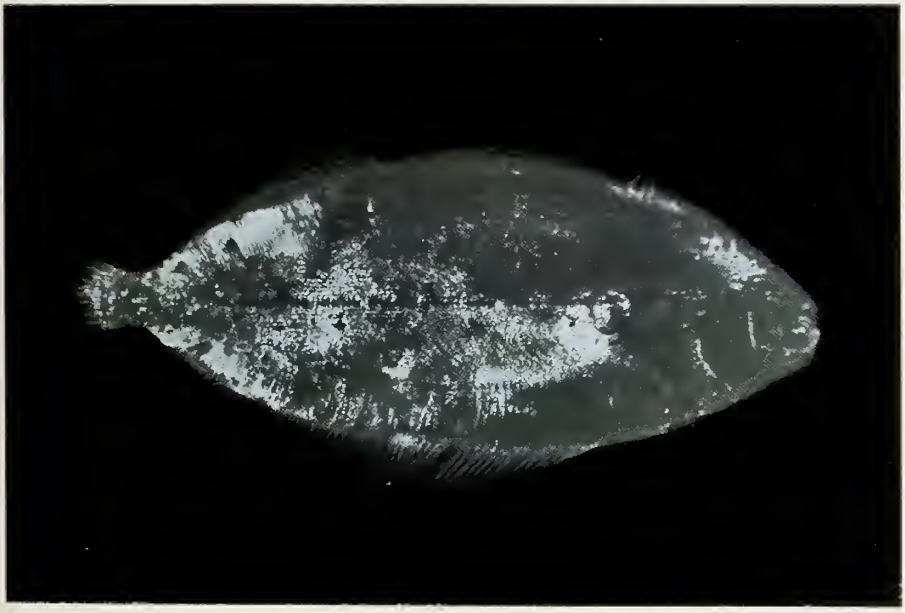
Changes in Pigmentation.—A few anaerobie bacteria are able to produce a red color in the absence of air. In all other cases pigmentation eeases under such conditions. High temperatures, close to the maximum endurable by the various pigment produeing speeies, give also colorless growths. The green color of *Bact. fluorescens* appears, as a rule, only in media of alkaline reaction; the sky-blue pigment of *B. cyanogenes* is deepened by aeids, as was said before. *B. prodigiosum* grows yellowish-red on alkaline, bluish-red on acid substrates. The red color of *B. erythrogenes* is formed only in the dark, while the pigments produced by certain molds become more intense under the influenee of light. Nutrition, especially presence or absence of certain salts, plays also its rôle in this case, as does spontaneous variability, so general in bacterial life.

White strains of *Bact. prodigiosum* and *syncyaneum* are very frequent; they should eertainly not be classed as distinet speeies, as was done repeatedly. It is still less appropriate to eonsider small differences in the tints of pigmentation as species marks. Natural variability and environmental conditions are usually responsible for such differences. Changes from white to yellow and to orange are very frequent among Mierococci. Old cultures of yellow rods often turn white; on the other hand, gradual changes are known to occur from the white *Bact. coli* to yellow rods. *Bact. fluorescens* as well as its counterpart *Bact. putidum*, which does not liquefy gelatin, but is otherwise very similar to the first-named organism, display their parallelism also in producing brown varieties, which in the latter ease can not be sharply distinguished from those of *Bact. syncyaneum*. A close relationship between these species is very probable. The same holds true concerning a great number of small motile liquefying rods producing a red pigment, which are sometimes classed as separate speeies, although there is much greater probability that they are merely varieties of *Bact. prodigiosum*. The reddish surfaee growth charaeteristic of Camembert, Brie, and of some other kinds of French cheese is only partly due to the presence of bacteria and molds of red color. Some white and yellowish species have been isolated from such material whieh produce this particular eoloration by symbiotie action.

Phosphorescence.—Produetion of light by plants and animals is of fairly frequent occurrence. Molds and bacteria again aet along the same lines as do the higher organisms. The phosphorescence often notieeable

1. Milk changed by pigment bacteria

Bacterium erythrogenes	Micrococcus aurantiacus	Bacterium fluorescens	Bacterium syncyaneum	B. fluorescens var. bruneum



2. Phosphorescent bacteria growing on fish
photographed in their own light
$^1/_2$ nat. size

in decaying wood is caused by fungi, while that of seawater, of fish, and of other food is produced by bacteria. The photograph shown on Plate VI was made in the darkroom by 16 hours exposure to the dim light of the phosphorescent bacteria forming the bright patches on the fish. The light produced by pure cultures is, of course, somewhat stronger, and it was used by H. Molisch and others for making interesting photographic pictures of various objects.[1] This investigator has also recommended the use of such cultures for making lamps for miners, which would preclude any danger of explosion. Unfortunately, the phosphorescence even of very active cultures is rather weak. An area of one square meter covered by a good growth of such organisms would furnish no more than 1/1000 to 1/100 candle power.[2] Furthermore, phosphorescence is very easily lost in pure cultures; most stock cultures of this kind grow well, but do not emit any light. It becomes evident from this fact that this process is not of vital importance. The oxidation of various substances causes the phosphorescence, which can be produced experimentally by adding, instead of bacteria, oxidizing chemicals like peroxide of hydrogen, bromine water, etc. to sterile humus, decayed wood, alkaline fish broth, and similar easily oxidizable substances.[3]

Production of Heat.—Phosphorescent bacteria and fungi, like fireflies, are mainly objects of curiosity, but the production of heat by microorganisms is of much greater interest and practical importance. Combustion of various substances in the process of respiration is always the cause of an increase in temperature with the lowest as well as with the highest organisms. Wherever large amounts of organic matter accumulate, rapid propagation of fungi and bacteria will take place, which participate in the production of heat simultaneously with the surviving plant cells and with oxidizing enzymes present in the material.

If the water content is very high, as in milk, liquid manure, or in other solutions, the rise in temperature is hardly noticeable. In ripening cream, however, where the percentage of water is somewhat reduced, an increase of 1° to 1½° C. is usually to be recorded. A lower content of moisture leads to such well known results as are obtained with fodder in silos and with stable manure in hot-beds. And if still less water is present the temperature may reach degrees where the organic matter assumes more and more the character of coal and may become subject to spontaneous ignition.

[1] H. Molisch, "Leuchtende Pflanzen," 2. Aufl., 1912.

[2] Friedberger und Doepner, *Centralbl. f. Bakt.*, I. Abt. Orig., vol. 32, 1907, p. 1; A. Lode, *Centralbl. f. Bakt*, II. Abt., vol. 22, 1909, p. 421.

[3] Weitlaner, *Verhandlungen d. Zoolog.-Botan. Gesellschaft zu Wien*, vol. 61, 1911, p. 192; Gerretsen, *Centralbl. f. Bakt.*, II. Abt., vol. 52. 1920, p. 353.

Gradual Increase in Temperature.—The whole process can be divided for the sake of clearness into three phases, (1) increase in temperature up to 45° C., (2) from 45° to 70° C., (3) above 70° C. The first phase is to be accepted as a normal occurrence, the second one is of use in certain cases, the third one is always abnormal and dangerous on account of the possibility of spontaneous ignition. Leaves, green fodder, bran, hay, tobacco, etc. enter the first phase quite regularly as soon as sufficiently large quantities are brought together, so that the accumulating heat can not be dissipated by cooling off at the outside. The respiration of living plant cells produces the initial heat; later, oxidation caused by enzymes of dying and dead cells comes into play, supported to a varying degree by the respiration of bacteria and fungi. In some cases, for instance in curing tobacco, the respiration of plant cells and the activity of enzymes alone are of importance; in other cases, as in the rise of temperature in stored peat, bacteria and fungi are solely responsible, but as a rule all three factors contribute to the final effect. Under normal conditions the rise in temperature reaches and exceeds 40° C. only in rare cases. Low water content, as in hay and bran, expulsion of air by strong pressure, as in silage, tend to keep the oxidation within rather narrow limits.

If temperatures of 40° to 45° C. persist for several days, plant cells as well as most of the common microorganisms will die, but their enzymes will remain active, and there will be also a new growth of thermophilic bacteria, actinomycetes, and fungi. If circumstances are favorable, the temperature will continue to rise until 70° C. is reached. Then microorganisms as well as enzymes cease to work, but purely chemical reactions may now proceed with great rapidity in the hot material. Numerous so-called species of thermophilic bacteria have been described more or less incompletely, and it is not to be doubted that their number could be greatly reduced by critical tests.[1] Most of them produce endospores which guarantee their survival during periods of low temperature, when all vegetative cells may die. Naturally, also the thermophilic organisms can exert their influence only if enough oxygen, oxidizable matter, and water are present. In material like coal a rise in temperature is almost exclusively caused by chemical action, as for instance by the oxidation of iron sulfides.

Spontaneous Ignition.—If the temperature reaches 70° C. and rises above this point the danger of spontaneous ignition becomes acute. It goes without saying that this last phase is purely chemical. But how the ignition finally takes place is still a matter of dispute. As a result of

[1] A review of the thermophilic organisms was given by Ambroz in *Centralbl. f. Bakt.* I. Abt. Ref., vol. 48, 1910, pp. 257, 289.

various chemical reactions easily oxidizable substances like hydrogen, methane, as well as volatile organic substances are evolved, whose presence is indicated by the peculiar smell of such hot substances. The high temperature increases, of course, their affinity for oxygen. If the organic matter changes, as often happens, to a porous finely divided coal, these volatile substances are adsorbed by it and may be ignited to a slowly proceeding glow whenever oxygen is suddenly admitted in large quantities either by a strong wind or by the removal of the upper and outer layers. Such pyrophoric (that is fire-bearing) coal, however, was not always found in hay stacks which, nevertheless, did show spontaneous ignition. It seems as if in such cases the volatile oxidizable products, because they are not adsorbed, accumulate as gases, which may be blown out by the wind or may escape when the hay is taken down. In both cases the ignition is very sudden, almost like an explosion, which fact explains why this type of spontaneous ignition has been very little studied.

2. TRANSFORMATION OF ORGANIC SUBSTANCES

The transformation and decomposition of products and residues formed and left by higher plants and animals represents the main field of bacterial activity. As a result of physiological and biochemical investigations it is well known that numerous processes participate in the formation of the chemical compounds which are more or less essential for the life of the higher organisms. As time proceeds, analogous data will accumulate with regard to bacterial life. At present, however, only the main lines have been studied along which bacterial activities proceed. But enough is known to present a fairly complete and accurate survey of these processes, especially as far as they are of importance to the agriculturist.

From daily experience it is well known that many volatile substances are produced by bacteria and fungi whose chemical composition is rather incompletely known. Agreeable or offensive odors and flavors can be noticed quite generally, and frequently the nose permits the formulation of a more correct judgment in regard to the presence and activity of microorganisms, than does the eye or any other sense. The disagreeable smell of unclean dairy utensils, for instance, indicates their condition very clearly, and in judging milk, butter, and cheese, the determination of their flavors often gives more reliable results than are obtainable by chemical methods.

Putrefaction, Decay, Fermentation.—The presence or absence of offensive odors is also used as one of the foremost marks for making a

simple differentiation between the various modes of decomposition. This was the case especially in regard to the terms putrefaction and decay, and in books written some decades ago many details about putrid odors may be found which, however, did not lead to any clear insight into these processes. The term fermentation, on the other hand, was and is commonly applied to transformations connected with a noticeable evolution of gas. Since bacterial activities are better known, some authors have advocated reserving the term putrefaction for the anaerobic decomposition of nitrogenous substances, the word decay for aerobic nitrogen transformation, and fermentation for the destruction of carbonaceous compounds. But as is always the case when vague popular terms are introduced into scientific language, the new meanings attached to them are not generally accepted, and misunderstandings are the inevitable results. How inconsistently those denominations are applied is clearly demonstrated, for instance, by the use of expressions like alcoholic fermentation and urea fermentation. In the first case the non-nitrogenous end product of the process is mentioned, but in the second case the starting point, which is here a nitrogenous substance, is used for designation. Analogous terms, like acid fermentation, slimy fermentation, etc., are by no means better; and it is undoubtedly preferable to avoid all such vague expressions wherever clear scientific terms are available.

Nitrogen-Carbon Ratio.—The quantity and quality of nitrogenous and non-nitrogenous organic compounds are of foremost importance among the factors which determine the general course of bacterial action. The situation is very similar to that in animal feeding, where the economic success is dependent upon the proper relation between proteins and carbohydrates in the food. All other factors, such as the presence or absence of air, the degree of moisture, the reaction, etc., will also exert their influences, but it is the effect of the carbon-nitrogen ratio which deserves closest attention. If there are comparatively large quantities of carbohydrates, as in silage, manure, and milk, the metabolism of nitrogen takes quite another course than in those cases where only few and not easily accessible carbon compounds are present, as in soil. It depends pre-eminently upon the carbon supply whether nitrate is formed by bacteria or whether it is destroyed, whether nitrogen is fixed or whether it is liberated from its compounds. These facts will be discussed and explained on the following pages.

Enzymatic Action.—Many of the substances which are attacked by bacteria and fungi are insoluble in water, and it is therefore self-evident that their transformation must be due to enzymatic action, because only soluble substances can enter the bacterial and fungous cells. But also in the case of soluble substances enzymes are known to be of importance, and

it is by no means improbable that ultimately all bacterial action may be found to be caused by enzymes. This fact, however, should not be misinterpreted, as is sometimes done, by asserting that the enzymes be of greater interest and importanee than the microorganisms themselves. Enzymes are soluble eell produets specially fitted to start one or another chemical transformation, whose products are usually of value to the living cells. The latter are always of primary importance, beeause they produce the enzymes. The digestive processes in man and animal are also mostly the result of enzymatic action, but nobody will contest that this is only of secondary importance eompared with the primary funetion of the living organism.

The participation of enzymes in the various transformations is of great significance in regard to the *intensity* and long *duration* of these processes. A very small amount of rennet is sufficient to coagulate large quantities of milk (approximately 1 : 800,000) and the enzyme remains active long after the death of the calf from which it was taken. Analogous relations exist between bacteria and fungi and their enzymatic actions. Certain urea bacteria, for instance, produce enzymes which eonvert in one hour a quantity of urea into ammonia which is more than 1000 times heavier than the weight of the bacteria themselves. These enzymes also eontinue to act long after the baeteria have died, and the same behavior is to be noted with the enzymes aetive in silage, in storage butter, or in slow ripening cheese. When bacteria first start growing, the enzyme production is weak. It increases with the bacterial development; but after this has reached its height and the cells begin to die, the enzymes continue to accumulate. The result is that the maximum in the number of living microorganisms always precedes the maximum in enzymatic aetion. This relation is especially marked in cases where the enzymes remain inside of the living cells (so-called endo-enzymes), but it is also noticeable under the opposite eonditions, that is, when ectoenzymes are produced.

3. THE CYCLE OF NITROGEN

Because of the great eeonomic importanee of nitrogenous compounds, their transformations have been thoroughly studied by baeteriologists as well as by chemists. Numerous details have been gathered in the course of these investigations which will be discussed later, insofar as they are of general interest. At present the fundamental faces eoncerning the cycle of nitrogen will be eonsidered in order to get an accurate view of the whole subjeet.

The Phases of the Cycle of Nitrogen.—Nitrates and ammonia represent the sourees of nitrogen for the higher plants, whieh transform

them to amino acids, amides, and proteins by combining them with carbonaceous material, previously prepared from carbon dioxide and water. The organic nitrogenous compounds are used by the animals, and afterwards they are broken up and returned to their mineral state by bacteria and fungi. But microorganisms participate also in other transformations of nitrogen, of which eight or nine are fairly well known, while some others are still more or less in doubt. Figure 29 presents in schematic arrangement all these possibilities; full drawn lines indicate

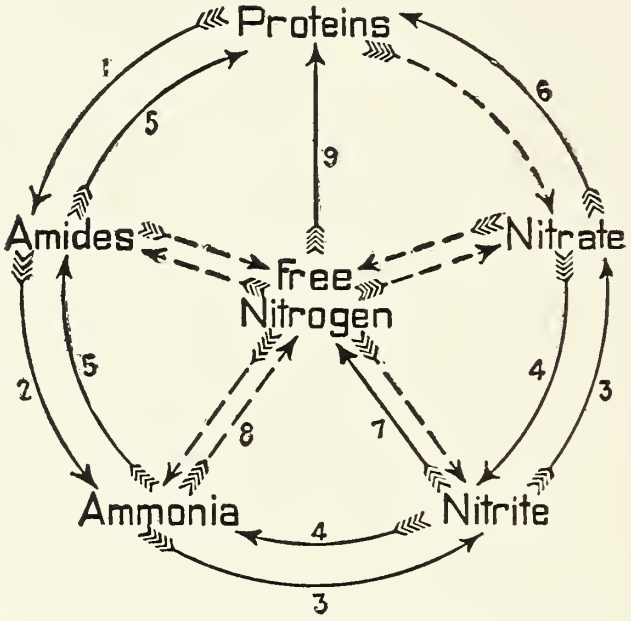


Fig. 29.—Cycle of nitrogen. (1) Protein decomposition, (2) Ammonia formation, (3) Nitrification, (4) Nitrate reduction, (5) Assimilation of ammonia and amino-nitrogen, (6) Assimilation of nitrates, (7) Denitrification, (8) Ammonia oxidation, (9) Nitrogen fixation.

well studied transformations, broken lines those not yet adequately known. Protein decomposition (1), ammonia formation (2), and nitrification (3) constitute the *normal* steps in the mineralization of nitrogenous compounds and represent the counterpart to the assimilation of nitrogen as performed by the higher plants.

Retrograde changes comprise the nitrate reduction (4) from nitrate to nitrite and to ammonia, and the assimilation of amino, ammonium and nitrate nitrogen (5 and 6). In performing these changes the microorganisms enter into competition with the cultivated plants, and may

sometimes become distinctly harmful to them. The change from nitrate to protein (6) which completes the cycle, is in lower as well as in higher plants not so direct as indicated in Fig. 29. It is very probable that in all these cases the transformation passes through the ammonia and amino stages, but especially with regard to the nitrate assimilating bacteria and fungi no details concerning the chemistry of the process are known at present.

Of greater importance than these more or less abnormal retrograde changes are the liberation and the fixation of free nitrogen. The *liberation* of nitrogen from nitrate or nitrite, known as denitrification (7), seems always to start from the latter compound; but the possibility remains that elementary nitrogen is also directly split off from nitrates. The oxidation of ammonia (8) to free nitrogen and water is another still problematical transformation, which seems to be partly responsible for the losses of elementary nitrogen from barnyard manure. The same holds true concerning the analogous decomposition of amides and amino acids. Nothing definite is known about this process, but there are indications that this transformation, too, is connected with the escape of free nitrogen from the manure pile.

The *fixation* of free nitrogen (9) leads, as far as is known, directly to protein compounds. In the root nodules of the leguminous plants, as well as in cultures of nitrogen fixing organisms isolated from the soil, no other products of assimilation have been found. But it is very probable that amides and amino acids occur as intermediate steps in these as in other cases. Some authors believe that there are also bacteria able to unite nitrogen and hydrogen to ammonia, or to oxidize nitrogen directly to nitric and nitrous acids. Well founded data in support of this opinion are not yet available; but in view of the fact that by chemical methods all three modes of nitrogen fixation can be performed, the possibility that microorganisms may act along the same lines should not be a-priori rejected.

Terminology.—The names as used for the different transformations of nitrogen are mostly applied in the manner just described. Sometimes the term "denitrification" is confounded with "nitrate reduction," or even with "nitrate assimilation," but such usage is not to be recommended. If merely the transformation or the loss of nitrates is ascertained, but not investigated which one of the three processes is actually involved, none of these specific terms should be used, but simply "transformation" or "loss of nitrate." To call the nitrogen fixation "nitrification" is another not infrequent mistake.

Likewise not justified is the use of the term "azofication" or "azotofication" instead of nitrogen fixation, because its real meaning is not

fixation, but "formation" of nitrogen, analogous to ammonification = formation of ammonia, and nitrification = formation of niter. Quite recently a new term "rhizofication" was proposed [1] to designate the nitrogen fixation occurring in the root nodules of the leguminous plants. It is to be hoped that it will not come into general use, because it is very incorrectly chosen; it means "root formation," not nitrogen fixation within the roots.[2]

Decomposition of Protein Substances.—On account of the varied compositions of the proteins their decomposition follows many different lines and leads to widely differing results. A rapid and complete mineralization of such substances is desirable in manure and in soil. In other cases, for instance in cheese ripening, only a partial change, some kind of pre-digestion of the proteins is attained by which they are partly transformed to amides, amino acids, and ammonium salts.

Higher organisms participate more or less actively in the first phase of the nitrogen cycle in direct competition with bacteria and related microorganisms. The proteins, produced by the plants from nitrate, carbon dioxide, and water, are widely used and transformed by the higher animals. As many animals live on, in, and from another, manifold changes may take place which, however, rarely lead below the first step, that is, below amides and amino acids. Close competition between higher and lower organisms is noticeable, for instance, in the intestines as well as in ripening cheese, provided that in the latter case mites and fly larvae are permitted to develop on its surface.

A multitude of protozoa, molds, yeasts, and bacteria is always ready to attack any protein substances not immediately used by higher plants and animals. Aerobic and anaerobic, psychrophilic and thermophilic microorganisms may become active. It is sometimes asserted in the literature that "genuine" putrefaction is caused exclusively by anaerobic bacteria. But because "genuine" putrefaction can not be defined exactly, this statement is of no consequence. It is to be admitted that certain strains of anaerobic bacteria, belonging to the group of *Bac. putrificus,* produce most offensive, putrid odors. However, decomposition of meat, usually accepted as an example of true putrefaction, is to a large extent due to the activity of *B. proteus,* an acrobic organism. Comparative tests made with representatives of both groups of bacteria gave the following results in regard to the lytic actions exerted upon the protein nitrogen within one month at 37° C.: [3]

[1] P. E. Brown, *Jour. Amer. Soc. Agron.*, vol. 13, 1921, p. 323.

[2] Derived from the Greek word ῥίζα (rhiza) = root, and the Latin word facere = make.

[3] H. Tissier *Annal. de l'Inst. Pasteur,* vol. 26, 1912, p. 522.

Percentage of Nitrogen made soluble	B. putrificus	B. proteus
Egg albumin	100	2
Blood albumin	86 to 91	66
Vegetable proteins	61 to 72	45
Meat	82 to 100	18
Milk	93 to 98	26
Lentils	33 to 53	3

Many other aerobic organisms, common in manure and in soil, such as *B. fluorescens* and related forms, as well as sporulating bacteria of the *subtilis-mesentericus* group, may participate in the protein decomposition, especially in symbiosis with anaerobic species which usually display their greatest activities in the initial steps of the process.

Various kinds of *enzymes* are active in these transformations. For attacking insoluble substances ecto-enzymes are produced, while soluble substances, able to enter the cell, are transformed by endo-enzymes. These bacterial enzymes are similar to those found in higher organisms, especially rennin, pepsin, trypsin, and erepsin, all active in animal digestion. Cooperation between animal or plant enzymes and those of bacterial origin is not infrequent. Milk enzymes and rennet combine their effects with those of the microorganisms in milk, butter, and cheese; plant enzymes are active in silage together with bacteria and fungi; animal enzymes enter the manure in the feces along with great numbers of microorganisms.

The first products of protein metabolism are usually of great nutritive value and are therefore repeatedly used by higher as well as by lower organisms. Only part of the nitrogen is changed into amino and ammonium nitrogen, and this delayed and incomplete mineralization is demonstrated by the slow and moderate fertilizing effects of substances like flesh meal, whale guano, barnyard and green manures.

Transformation of Amides and Amino Acids.—Certain amides and amino acids, especially asparagin, aspartic acid, alanin, leucin, and tyrosin, are still of fairly high nutritive value for numerous microorganisms, as was demonstrated for asparagin on Plate IV. The nitrogen present in such form is therefore not readily transformed into ammonia. This, however, is the case with those amides and amino acids which constitute the nitrogenous part of liquid manure, that is with urea, hippuric and uric acids.

The transformation of *urea* into ammonia is represented by the following formula:

$$CO(NH_2)_2 + 2\ H_2O = (NH_4)_2CO_3$$

Presence or absence of air is of no influence upon this transformation. The urea bacteria themselves grow better under aerobic than under anaerobic conditions; but as not their growth, but the production of the active enzyme called *urease,* is of significance, and the enzymatic action is, of course, independent of the presence of oxygen, rapid transformation of the urea takes place in liquid manure even in complete absence of air. Therefore, the practical usefulness of air-tight covers on tanks and pits used for storing liquid manure, is not to be explained by any beneficial effect upon the amide transformation as such, but merely as due to the protection afforded against the evaporation of ammonia. Various cocci and bacilli are known to be able to transform urea into ammonia; sometimes they are grouped into special genera, Urobacillus, Urococcus, and Urosarcina. But their activity can easily cease, and they are in fact not representatives of separate genera, but merely varieties of such common species as *B. proteus, coli, prodigiosus, fluorescens, erythrogenes.* There are a few species which temporarily may display a very great activity and a very pronounced adaptation, as is the case with the spore-forming *Bacillus* (*Urobacillus*) *Pasteuri,* but they, too, can live without urea. The ability to produce urease is not restricted to bacteria. Several fungi as well as higher plants follow the same lines; soy beans, for instance, are comparatively rich in urease.

The transformation of *hippuric acid* is generally slower than that of urea, and it is dependent on the presence of oxygen (in air, nitrate, or in sugar). Glycin and benzoic acid appear first, according to the formula

$$C_6H_5CO.NHCH_2COOH + H_2O = NH_2CH_2COOH + C_6H_5COOH$$

Usually the same organisms, bacteria as well as fungi, perform this and also the next transformation which leads to ammonia. They are in part identical with those which hydrolyze the urea.

Still a little more complicated and less rapid is the transformation of *uric acid* which is at first changed to allantoin and then to urea.

$$\text{I. } 2\ C_5H_4N_4O_3 + O_2 + 2\ H_2O = 2\ CO_2 + 2\ C_4H_6N_4O_3$$

$$\text{II. } C_4H_6N_4O_3 + O_2 + H_2O = 2\ CO_2 + 2\ CO(NH_2)_2$$

Some species stop at urea, while others continue their work; *B. fluorescens,* other acrobic short rods, and several molds are such organisms. A sporulating bacterial species named *Bac. acidi urici,* may become active under anaerobic conditions.[1]

The transformation of *cyanamid* passes likewise through urea. The

[1] Liebert, *Proc. Acad. Amsterdam,* vol. 17., 1909.

1. Ammonia production and bacterial growth
in urea broth in peptone solution

2. Transformation of ammonia and nitrate
in soil extract containing 0.05 % di-potassium phosphate and
0.1 % ammonium sulfate + chalk or 0.1 % nitrate or 0.1 % nitrate + 1 % sodium citrate
Nitrification — Nitrate assimilation, Denitrification

chemical substance contained in the fertilizer called cyanamid or nitrolime is calcium cyanamid. This separates quickly in moist soil into calcium hydroxide and cyanamid. The latter is then rapidly changed by soil colloids to urea, and this by bacteria to ammonium carbonate.

$$\text{I. } CN.NCa + 2\,H_2O = CN.NH_2 + Ca(OH)_2$$

$$\text{II. } CN.NH_2 + H_2O = CO(NH_2)_2$$

$$\text{III. } CO(NH_2)_2 + 2\,H_2O = (NH_4)_2CO_3$$

No bacteria, but some fungi are known to be able to attack cyanamid directly; under natural conditions, however, the quicker action of soil colloids prevents their becoming active.[1] The urea derived from cyanamid is not transformed by the typical urea bacteria, but by various other kinds, which seem to be less susceptible to the poisonous character of the intact cyanamid and of some of the by-products present in the commercial fertilizer.

Ammonia Formation.—The comparatively simple hydrolyzing processes occurring in liquid manure and with cyanamid transfer nearly all nitrogen into ammonia, and only very little of it is used for sustaining the life of the enzyme producing microorganisms. With proteins the opposite relation is to be observed; a luxuriant growth of bacteria and fungi takes place, but very little ammonia is liberated. In Fig. 1, Plate VII, two culture flasks are shown, one containing beef broth to which 10 per cent urea had been added, the other filled with peptone solution; both flasks were inoculated with a few drops of a manure infusion. The urea broth remained clear, no bacterial growth is visible, whereas the peptone solution is turbid and covered with a grayish-yellowish film. But contrary to this weak or luxuriant development of bacteria, much ammonia was produced in the first and very little in the second flask, as is indicated by the strong color reaction or its absence on the strips of turmeric paper placed between the cotton stopper and neck of the flasks.

The cause of these opposite results is to be sought in both the nitrogenous and carbonaceous components of these two classes of nitrogen compounds. The proteins are, as a rule, good sources of nitrogen as well as of carbon, while most of the amides and amino acids are deficient in one or the other direction or in both. In the presence of large quantities of easily accessible carbonaceous compounds, such as carbohydrates, glycerol, and similar substances, those amides and amino acids are also very readily assimilated by numerous bacteria and fungi, and the formation of ammonia is much reduced.

[1] Löhnis, *Zeitschr. f. Gärungsphysiologie*, vol. 5, 1914, p. 16.

Ultimately, however, all organic nitrogen will be ammonified, and even such resistant or poisonous substances as chitin, quinine, strychnin, morphin and nicotin, or the toxins produced by pathogenic bacteria are no exceptions to this rule.[1] The regular course of the nitrogen cycle may be delayed, but it will never be broken. As was mentioned above, very numerous species are collectively active under anaerobic and under aerobic conditions, at high as well as at low temperatures. Ammonification is still noticeable, for instance, in the soil when its temperature is close to the freezing point.

Nitrification.—The transformation of ammonia into nitrite and nitrate represents the last step in the mineralization of nitrogen compounds. Two groups of bacteria participate in this process. At first the nitrite bacteria become active according to the following formula:

$$2\ NH_3+3\ O_2=2\ HNO_2+2\ H_2O$$

Then the nitrate bacteria complete the oxidation:

$$2\ HNO_2+O_2=2\ HNO_3$$

Lime and other basic substances of the soil neutralize the acids formed. Whether or not organisms exist which are able to oxidize ammonia, or perhaps even organic nitrogenous compounds, directly to nitrate, is not known at present. Some authors have advanced such opinions, but no convincing proof has been furnished. The laboratory air contains, as a rule, small amounts of nitrous acid which are readily absorbed by slightly alkaline solutions if these are kept for a few weeks. Therefore a little nitrite is to be found in nearly every liquid culture, but this should not be accepted as valid proof of nitrite formation.

Pure cultures of nitrifying bacteria were first obtained by the Russian bacteriologist S. Winogradsky in 1890; but the existence of the two groups of organisms was known before that time, and numerous data in regard to their behavior had been collected in earlier years by Schlösing, Müntz, and Dehérain in France, and by Warington and Frankland in England. One of the peculiarities of the nitrifying bacteria is their great sensitiveness to large quantities of soluble organic substances. The in-

[1] Concerning the ammonification of chitin see W. Benecke, *Bot. Zeitg.*, I. Abt. vol. 63, 1905, p. 227, and K. Störmer, *Jahresber. d. Ver. f. angew. Bot.*, vol. 5, 1907; p. 128, concerning quinine, morphin, strychnin, Soyka, *Arch. f. Hyg.*, vol. 2, 1884, p. 281; concerning nicotin, J. Behrens, *Centralbl. f. Bakt.*, II. Abt., vol. 7, 1901, p. 1; concerning bacterial toxins, Charrin et Mangin, *Compt. rend. Soc. Biol.*, vol. 49, 1897, p. 545, and E. Metchnikoff, l. c., p. 592.

organic respiration supplies them with energy for assimilating carbon dioxide, and like the green plants they are unable to grow in substrates containing considerable quantities of carbohydrates, organic salts, or organic nitrogenous compounds. Gelatin and ordinary agar, therefore, do not permit growth, and platings could be made successfully only after W. Kühne had introduced silica jelly for such purposes. Later it was shown by Beijerinck that agar can also be used after it is freed from all soluble material by careful washing.

The nitrite bacteria grow either as small, oval, motile rods with polar flagella (Fig. 5, Plate II), or in larger, immotile, globular form. They were named by Winogradsky *Nitrosomonas* and *Nitrosococcus*, respectively, but it is very probable that the coccoid type represents merely the growth of regenerative bodies of *Nitrosomonas*. The nitrate bacteria are short, immotile rods, which have received the name *Nitrobacter*. Investigations upon their sensitiveness to organic substances and ammonia were made by Winogradsky in cooperation with Omelianski.[1] The following quantities were found to inhibit growth and action of the nitrifying organisms in alkaline solutions:

	Pepton Per Cent	Asparagin Per Cent	Urea Per Cent	Glucose Per Cent	Ammonia Per Cent
Nitrosomonas	0.2	0.3	?	0.2	—
Nitrobacter	1.25	0.5 to 1.0	1	0.2 to 0.3	0.015

Winogradsky drew from these findings two conclusions which were generally accepted and widely copied in bacteriological textbooks. They read: All soluble organic substances must be decomposed in the soil before nitrification can take place, and all ammonia must be first converted into nitrite before *Nitrobacter* can begin its activity. However, these generalizations were not supported by the facts observed.[2] Under normal conditions the soil solution is nearly neutral and does not contain such large quantities of soluble organic substances as were found to be detrimental, and *Nitrobacter* is very sensitive only to free ammonia, but not to such ammonia salts as are present in the soil. Nitrite and nitrate formation proceed, as a rule, simultaneously in the soil, and the organic substances of the soil, that is, humus compounds, are not detrimental but usually very favorable to the nitrifying organisms, so that in most cases the more humus is present in a soil the more active are its organisms. Highly acid peat soils are to be excepted, of course, not so much on account of their organic substances, as because of the fact

[1] Winogradsky und Omelianski, *Centralbl. f. Bakt.*, II. Abt., vol. 5, 1899, p. 436.

[2] Löhnis, *Centralbl. f. Bakt.*, II. Abt., vol. 13, 1904, p. 706; Fred and Davenport, *Soil Science*, vol. 11, 1921, p. 389.

that they do not contain a sufficient amount of basic compounds necessary to neutralize the nitric and nitrous acids, and not enough oxygen to permit rapid oxidation.

In barnyard manure, as well as in liquid manure, only little or no nitrification takes place, because the presence of soluble organic substances and the restricted supply of air both exert their adverse influences. But if stable manure is exposed to the air in thin layers a marked nitrification may establish itself.[1]

Some authors have thought that the nitrification of ammonia be regularly connected with losses in free nitrogen, usually estimated at about 10 per cent. If unfavorable conditions prevail, and therefore ammonium nitrite can accumulate in considerable quantities, such losses are indeed possible according to the formula:

$$(NH_4)NO_2 = 2\ H_2O + N_2$$

Usually, however, this reaction is of no importance, and it has been repeatedly ascertained in exact experiments that the nitrification as such does not entail any losses. The nitrogen requirements of the nitrifying organisms are so low that for all practical purposes it can be assumed that 100 parts of ammonium nitrogen will give 100 parts of nitrate nitrogen, provided that this transformation is not disturbed by antagonistic actions.

Nitrate Reduction.—While nitrification is the work of probably not more than two groups of highly specialized organisms, there is, on the other hand, a great number of bacteria and fungi capable of causing the opposite reaction. Some of them reduce the nitrate only to nitrite, others confine their action to the reduction of nitrite to ammonia, but most of them perform the complete retrograde transformation from nitrate to ammonia. This function, however, is rather inconstant and may be present or absent among closely related varieties of one species. It can therefore not be used for diagnostic purposes.

Easily oxidizable substances of the soil, first of all humus compounds, may participate actively in the nitrate reduction. In peat soils it sometimes happens that the biological nitrate reduction is completely replaced by this purely chemical reaction. But even if microorganisms are active, it is not always their need of oxygen which is responsible for the reduction. Frequently products of their metabolism are easily oxidized and liable to reduce the nitrate. Accordingly, the transformation of nitrate to nitrite often takes place in the presence of air, but the second step from nitrite to ammonia requires always more or less anaerobic conditions.

[1] Niklewski, *Centralbl. f. Bakt.*, II. Abt., vol. 26, 1910, p. 388.

These facts explain why nitrate reduction plays a conspicuous rôle only in peaty, swampy, water logged soils. Heavy rains, inundations, and insufficient drainage may establish similar conditions in other soils too, but in general the nitrate reduction is not of very great importance. As soon as air again pervades the soil the ammonia is once more quickly nitrified.

Useful application of nitrate reduction is made in the cheese industry when saltpeter is added to the curd in order to prevent gassiness of the cheese produced. If no nitrate is added the possibility exists that part of the lactose will be used by certain bacteria as an oxygen supply, and the liberated hydrogen and carbon dioxide will cause the deformation of the cheese. Nitrate, however, prevents this fermentation by serving as an easily accessible source of oxygen; the ammonia formed is promptly changed into harmless organic salts.

Assimilation of Amino, Ammonium, and Nitrate Nitrogen.—The presence or absence of oxygen determines whether nitrification or nitrate reduction will take place, and it is the absence or the presence of large amounts of easily accessible organic carbon compounds which decides whether ammonia will be formed and nitrified, or whether microorganisms will assimilate nitrate, ammonium, and amino nitrogen. Because soluble organic compounds are very common in nature, it is self-evident that this assimilation of the simpler nitrogen compounds is no rare occurrence, and therefore of greater importance than the nitrate reduction. It was pointed out before (p. 43 and Plate IV) that a relatively poor source of nitrogen, such as urea, ammonia, and nitrate, becomes accessible to most of the microorganisms only if a good source of carbon is simultaneously present. Urea is quickly and completely transformed into ammonia in soil where very little soluble organic substances are to be found, but in barnyard manure 30 to 70 per cent of the urea nitrogen is assimilated and transformed into bacterial proteins. If a large quantity of straw or of fresh manure is plowed under a few days or weeks before a new crop is planted, it will not show any marked fertilizing, but often a distinctly disadvantageous, effect, because the carbohydrates and organic salts contained therein will enable numerous microorganisms to assimilate ammonium and nitrates, previously formed in the soil.

The assimilation of amino, ammonium, and nitrate nitrogen is performed by acrobic organisms. As a rule, the nitrogen of amides and amino acids is more readily utilized than that of ammonium salts, and this is generally better assimilated than nitrate nitrogen. Under average soil conditions very little nitrate nitrogen becomes inaccessible to the roots of the higher plants on account of the interference of nitrate assimilating bacteria and fungi. But almost without exception a more or less marked

assimilation of ammonium salts takes place in all soils, even if no fresh manure or straw has been added. The fertilizing effect of ammonium sulfate is therefore frequently more or less inferior to that of sodium nitrate. Especially in light soils, where the physical and chemical absorption of ammonia is not very strong, sometimes the biologic fixation of ammonia by assimilating microorganisms may become very marked.

Repeatedly the statement has been made in the literature that the assimilation of ammonium nitrogen is done mostly by molds, while that of nitrate nitrogen is declared to be due to bacterial activity. This generalization, however, is not tenable. Whether fungi or bacteria will predominate depends mostly on the source of carbon and on the reaction of the substrate.[1] Ammonium carbonate, for instance, gives only bacterial growth. Organic ammonium salts, but also nitrate, are assimilated mostly by molds if glucose is present, because this is easily converted into acids. Sulfates and chlorides of ammonium stimulate the development of fungous growth, because they are physiologically acid, that is, the acids are left and make the substrate sour when the ammonium is used. But in the presence of certain carbon compounds bacteria, too, may display vigorous growth, as was shown on Plate IV in regard to ammonium sulfate and glycerol.

Whether the assimilation is done by bacteria or by molds is of considerable importance, because the renewed mineralization of the converted nitrogen proceeds, as a rule, fairly rapidly if bacterial cells and their products are present, whereas spores and conidia of molds are much more resistant. Young spore-free mycelia, however, behave like bacteria. Comparative tests have shown[2] that 20 to 40 per cent of such bacterial nitrogen was nitrified in soil, where at the same time and under analogous conditions only 4 to 8 per cent was mineralized if mold growth, rich in spores, was used as source of nitrogen. This slow and incomplete mineralization of bacterial and fungous cells makes the assimilation of amino, ammonium, and nitrate nitrogen by microorganisms a distinctly disadvantageous process, which should be avoided as far as possible. Proper rotting of stable manure and of straw before their incorporation into the soil is helpful in this respect.

Liberation of Nitrogen.—Losses of nitrogen by purely chemical reactions may occur, if amides and ammonia have an opportunity to react with free nitrous acid according to the formulae:

$$CO(NH_2)_2 + 2\,HNO_2 = 2\,N_2 + 3\,H_2O + CO_2$$

$$NH_3 + HNO_2 = N_2 + 2\,H_2O$$

[1] St. Bierema, *Centralbl. f. Bakt.*, II. Abt., vol. 23, 1909, p. 672.

[2] Bierema, l. c.

These processes play their rôles probably in the manure pile, as well as in certain peat soils, where as a result of improper handling (excessive liming, especially) considerable quantities of the peat nitrogen are sometimes transformed into ammonium nitrite and more or less completely liberated.[1] Very large applications of ammonium sulfate may cause similar losses in normal soils, too, as was demonstrated by Th. Schlösing,[2] who found that 4 to 8 per cent nitrogen disappeared when ammonium sulfate was used in quantities equivalent to 4000 to 7000 lbs. N per acre, that is, about 100 times as much as is applied normally.

Among the other ways in which nitrogen may be liberated, the decomposition of amides and of ammonia seems to be of importance especially in regard to the transformation of nitrogen in barnyard manure. But, as was pointed out above, no definite data in these respects are available at present. The only well-known mode of liberation of nitrogen by bacteria is the so-called dentrification, that is, the decomposition of nitrates and nitrites under anaerobic conditions with liberation of free nitrogen.

Denitrification.—As is the case with nitrate reduction, there are two possibilities of denitrification. Either a "direct" denitrification is performed by bacteria and their enzymes, or an "indirect" denitrification takes place, caused by hydrogen and other easily oxidizable substances resulting from the decomposition of organic substances. Furthermore, certain sulfur bacteria may act as denitrifiers; but this is a rather rare case and therefore not of general interest. It will be explained in Chapter VII, 5.

Nearly all the nitrogen split off by direct denitrification appears in the free, elementary form, while indirect denitrification gives rise to smaller or larger quantities of nitric oxide (N_2O_2) and nitrous oxide (N_2O) besides free nitrogen. Naturally both processes occur often simultaneously. The indirect denitrification is, of course, of no importance to the organisms whose metabolic products are oxidized, but the direct denitrification enables otherwise aerobic bacteria to live under strictly anaerobic conditions. The oxygen taken from nitrate, nitrite, and from nitrous oxide replaces the free oxygen in the process of respiration, according to the following equations:

$$2\,KNO_3 + 2\,C.. = N_2O + K_2CO_3 + CO_2$$

$$2\,KNO_2 + C.. = N_2O + K_2CO_3$$

$$2\,N_2O + C.. = 2\,N_2 + CO_2$$

[1] Th. Arnd, *Landw. Jahrb.*, vol. 47, 1914, p. 371.

[2] Th. Schlösing, *Compt. rend. Acad. Paris*, vol. 109, 1889, p. 884.

Nitrite and nitrous oxide are frequently, though not always, traceable as intermediate produets.

The number of denitrifying bacteria described thus far is rather large, but it is beyond doubt that many of these so-called species are merely varieties of other speeies which usually do not denitrify. Various authors baptized their respeetive cultures *Bacterium* or *Bacillus denitrificans* with or without additional numbers I, II, III, etc., which has led, of course, to much confusion. *Bacterium Stutzeri* Lehm. et Neum. is a common and easily recognized denitrifier. *Bact. fluorescens, putidum,* and *radiobacter* are sometimes inclined to act in the same manner. *Bac. denitrificans agilis,* first isolated by Ampola and Garino in Italy, is, for instance, a denitrifying variety of *Bact. radiobacter.* Long continued cultivation in the absence of nitrate and in the presence of air usually leads to the disappearance of this character, which may be newly acquired, on the other hand, under reversed conditions.

If no other nitrogen compounds are present in the substrate, part of the nitrate will, of eourse, be assimilated. Under completely anaerobic conditions approximately 95 to 98 per cent of the nitrate nitrogen is split off, and only the remainder is assimilated. The more free oxygen will find access to the substrate, the more nitrogen will be assimilated, and less nitrogen will escape; with full aeration the nitrate assimilation replaces the denitrification entirely.

The culture vessels shown in Fig. 2, Plate VII, may illustrate these relations. The first flask to the left contains a shallow layer of soil extract + ammonium-sulfate + ehalk; accordingly, nitrification took place. The cylinder next to it contains a deep layer of soil extract + nitrate; no transformation took place, because no suitable carbon compound was present. To the right a flask is shown which contains a shallow layer of soil extract + nitrate + sodium citrate; on account of the aerobic conditions the nitrate was assimilated by bacteria growing in the solution and as a film upon its surface. The same solution placed in a deep layer in a eylinder (to the right), exhibits very little bacterial growth, but a thick white scum is formed by the bubbles of liberated nitrogen.

The conditions under which denitrification takes place are (1) Presence of nitrate, (2) Presence of suitable organic carbon compounds, (3) Absence of free oxygen. Of course, sufficient moisture, adequate temperature, the necessary mineral salts, etc., must also be available. But the three conditions first mentioned explain at once why, with proper management, the losses due to denitrification in barnyard manure and in soil can be kept within fairly narrow limits. A properly kept manure pile does not offer a good substrate for nitrification, which would have to

precede any denitrification. And in field soils, as a rule, the amount of organic substances is so low and the aeration so strong that very little denitrification can take place. Very wet soils and an excess of organic substances naturally will change the situation. Under such conditions great losses may indeed be caused by denitrifying bacteria.

Nitrogen Fixation.—The only well known manner of biological nitrogen fixation is the assimilation of free nitrogen by certain bacteria, fungi and algae. No higher organism is able to build up proteins from elementary nitrogen, and in view of the losses in nitrogen compounds caused by the liberation of free nitrogen, this peculiar capability of some microorganisms is of fundamental importance for the continual restoration of the equilibrium of compound nitrogen available for lower as well as for higher organisms. During the last ten or twelve years various chemical methods have been developed which make it possible to produce amids (cyanamid), ammonia, or nitrate from the nitrogen of the air. But the nitrogen assimilation performed by bacteria is undoubtedly of greater importance, because much more nitrogen is fixed in this manner and at a much lower cost than by any technical process.

Nitrogen assimilation takes place only if large quantities of easily accessible carbonaceous substances are available. If this source of energy is missing, fixation of free nitrogen becomes impossible, and the microorganisms concerned live like all others on various nitrogen compounds. The following types of biological nitrogen fixation are known at present: (1) Nitrogen assimilation by the root nodule bacteria of leguminous plants, (2) nitrogen assimiiation by microorganisms living on and in the roots of other, non-leguminous plants, (3) nitrogen assimilation by bacteria living in the leaves of certain tropical plants, (4) nitrogen assimilation by various bacteria, fungi, and algae in the soil. In the first three cases higher and lower organisms live in close symbiosis, while there is no symbiosis in the last case, or only one among microorganisms.

Nitrogen Fixation by Leguminous Plants.—As stated in the discussion of the history of bacteriology (p. 5), about 2000 years ago Roman agricultural writers were fully aware of the fertilizing effect which may be realized from the cultivation of leguminous plants. But in the Far East (China and Japan) the same knowledge had gained a foothold at still earlier times, as proved by the use of legumes for green manuring since ancient periods.[1] The same practice became firmly established in European agriculture in the course of the nineteenth century. And the more it is applied to American farming the more will beneficial results be obtained.

[1] F. H. King, "Farmers of Forty Centuries."

In 1838 the first exact experiments were made by the French chemist Boussingault, which indicated that it is the fixation of nitrogen from the air which exerts the fertilizing effect upon the growth of leguminous plants.[1] Since then it has been generally acknowledged by European farmers that the most practical and economical means of supplying the farm with nitrogen is by including legumes in every crop rotation. Most scientists, however, insisted on more rigorous tests, but these gave mostly negative results during the next decades. It was considered necessary to heat the soil thoroughly before using it for such experiments, and some very careful investigators even covered the soil in their pots with thick layers of wax in order to prevent absorption of ammonia from the air. Bacterial life was, of course, impossible under such circumstances. But as early as 1851 another French scientist, named Roy, demonstrated that the nitrogen gains entrance to the legumes only through the roots, not through the leaves, and ten years later Bretschneider discovered that it was the high temperature to which the soils were exposed, which made nitrogen fixation impossible. Furthermore, in 1858 it was noticed by Lachmann that the peculiar nodules, characteristic of the roots of leguminous plants, are caused by the invasion of motile bacteria, which remain therein as long as the plant lives, and he refers to the opinion shared by many agriculturists of his time that these nodules are the organs of nitrogen fixation. It is beyond doubt that a correct insight would have been reached quickly if all results obtained had been properly considered and correlated. But the conclusion that nitrogen fixation takes place in the root nodules as a result of bacterial activity was evidently still too new and too strange to the leading scientists of that time.

Nevertheless, more and more positive findings were gathered. Several German farmers, who had obtained splendid results on very poor sandy soils merely by the cultivation of leguminous plants, stated once more and very firmly that nitrogen fixation must be the cause of this beneficial effect. That they and the earlier investigators were perfectly right was finally decided by thorough experiments made in the eighties of last century by Atwater in America, by Hellriegel and Wilfarth in Germany, and by Lawes and Gilbert in England. Not all authors, however, were ready to be convinced by the facts recorded. The "new-fangled bacteria hypothesis" was still frequently ridiculed, and even to-day it happens from time to time that far-fetched and quite unwarranted theories are put forward in order to displace those now firmly established results. T. Jamieson in Scotland ascribed, for instance, only a few years ago the

[1] Complete references to this historical summary are given in Löhnis, "Handbuch der landwirtschaftlichen Bakteriologie," 1910, pp. 646–650.

ability of nitrogen assimilation to special hair-like protrusions of leguminous and other plants, although it had been known since Roy reported upon his experiments in 1851 that the fixation and transformation of nitrogen takes place in the roots, not in the aerial parts of the legumes.

Nodule Bacteria of Leguminous Plants.—The results obtained by the American, German, and British chemists mentioned above, were completed by bacteriological experiments made by M. W. Beijerinck in Delft and published in 1888–1891. He was the first to succeed in obtaining pure cultures of the nodule bacteria, and was able to demonstrate their ability to produce root nodules and to assimilate nitrogen from the air. Equally valuable investigations on the same subject were made simultaneously by A. Prazmowski in Poland; his results were in complete agreement with those of the Dutch bacteriologist. But growth and activity of nodule bacteria in pure culture are not always satisfactory. According to their adaptation to symbiotic life they must find special environmental conditions, otherwise they will not fully display their abilities. Their growth on the substrates ordinarily used in the laboratories is very slight, and the same holds true in regard to nitrogen fixation. Several investigators could not discover any nitrogen assimilation in their experiments; others, however, secured positive results. Usually these gains in nitrogen are low (approximately 2 to 3 mg. N per 100 cc. of a 1 per cent sugar or mannite solution), but if an arrangement is made by which it becomes possible to add at short intervals only small amounts of nutrient solution and to remove promptly the metabolic products from the bacterial growth, as is the case in the plant, the nitrogen fixation shows a marked increase (from 2 to 3 to 6 to 12 mg.). Undoubtedly, the bacterial activity within the root nodules is still more efficient and more economical, but the results suffice to prove that the bacteria themselves fix the nitrogen, and that it is erroneous to assume, as has been done repeatedly, that the bacteria only "stimulate" the green plant in some mysterious manner so that the latter acquire the ability to assimilate free nitrogen.

Beijerinck chose as scientific name of the nodule bacteria the designation *Bacillus radicicola*. According to the rules of scientific nomenclature this species name must be retained, although instead of Bacillus frequently the generic names Bacterium and Pseudomonas were and are used, since no uniform usage has been established in this respect, as was discussed in Chapter III. All nodule bacteria are temporarily motile, but the mode of flagellation was for a long time a matter of dispute. Their gonidia are always monotrichous, as was first observed by Beijerinck. But full grown nodule bacteria exhibit two types of flagel-

lation. Those occurring in Trifolium, Medicago, Vicia, Pisum, and other cultivated leguminous plants of European origin are peritrichous, while those living in the roots of Soja, Vigna, Lespedeza, and other natives of Asia have polar flagella.[1] It is still doubtful whether changes from one to the other type are possible or not; the majority of observations made in this respect are against such a possibility.

Two years after Beijerinck had published his findings, a German botanist, A. B. Frank, gave a quite different description of what he erroneously believed to be the nodule producing organism, and proposed the name *Rhizobium leguminosarum* for his bacterium which was characterized by a yellow pigment. It is, of course, very incorrect to use this name instead of *B. radicicola* Beij. for the genuine nodule organism, as was done repeatedly.

From soil another species was isolated by Beijerinck which resembles *B. radicicola* in many respects rather closely, but which does not produce root nodules. It was named *Bacillus* (or *Bacterium*) *radiobacter*. Its growth on artificial substrates is somewhat better than that of the nodule bacteria, and because it also often invades the root nodules it was repeatedly isolated from there and confounded with the real nodule organism.[2] Certain varieties of *B. radiobacter* indicate a relationship to *B. coli* and to certain other bacteria common in soil as well as in milk. Branching, which was frequently noticed with nodule bacteria, can be observed in all of them, and it can not be accepted as a reason to place *B. radicicola* far apart from those related forms, as was repeatedly recommended. In Chapter I it was emphasized that branching is by no means so rare among bacteria as was formerly thought.

Nitrogen Fixation in the Roots of Non-leguminous Plants.—Similar nodules as are regularly found on the roots of legumes, have also been noted with representatives of several other groups of higher plants. In part they are purely pathologic, caused by the intrusion of parasitic organisms. But with certain plants they are a constant and characteristic feature, just as with the legumes. Whether they perform analogous functions is still a matter of dispute. Some positive indications were obtained, but very often even the efforts to cultivate the causative organisms have failed entirely. The conspicuous indifference displayed by these plants toward the nitrogen content of the soil makes further experiments very desirable. Some members of this group appear fitted for improving otherwise uncultivated stretches of land.

Such nodule bearing plants, mostly trees and shrubs, are the alder

[1] F. LÖHNIS and R. HANSEN, *Jour. Agric. Research*, vol. 20, No. 7, 1921, p. 543; I. SHUNK, *Jour. Bact.*, vol. 6, No. 2, 1921, p. 239.

[2] LÖHNIS and HANSEN, l. c.

(Alnus), several Elaeagnaceae (Elaeagnus, Hippophae, and Lepargyrea), Podocarpus, Myrica Gale, Comptonia peregrina, Ceanothus, Coriaria, and Cycas species.[1] In the last-named case the nodules are inhabited not only by nitrogen fixing bacteria but also by blue green algae (Nostoc or Anabaena). From the other hosts several organisms have been isolated which either resemble *B. radicicola* or exhibit more the characters of an Actinomyces.

Various cruciferous plants have also been supposed to assimilate free nitrogen, although it is more probable that they merely exhaust very thoroughly the supply of nitrogen compounds present in the soil. Nevertheless, further investigations are needed. An Italian author has stated that nitrogen fixing bacteria, related to *B. radicicola*, are active in the roots of such plants.[2]

Many plants whose natural habitats are in more or less acid soils rich in humus, such as the Ericaceae, Conifers, and others, are known to have various fungi growing on and in their roots, forming a so-called *mycorrhiza*. Despite numerous investigations, full light has not yet been shed upon the physiological value of this symbiosis, but it is certain that different functions are to be considered, and it is probable that among them nitrogen fixation plays its rôle.[3] Several *Phoma* species have been found to be capable of assimilating free nitrogen.[4]

Nitrogen Fixation in the Leaves of Tropical Plants.—Another interesting type of symbiosis between bacteria and higher plants was more recently discovered in several tropical genera (Pavetta, Psychotria, Ardisia, Spathodea, etc.) some of which have been used since ancient times, like the legumes, for green manuring. The bacteria again resemble in morphological and cultural characters *B. radicicola* to some extent, but instead of producing nodules at the roots, they establish themselves in the leaves, which are everywhere or only at the edges covered by small bead-like nodules filled with bacteria. Unlike *B. radicicola*, which does not invade the stems and seeds of its hosts, this is done by those organisms. There is a great number of them always to be found in the seeds, and in view of the difficulty with which the leaves would otherwise be reached by the bacteria, such permanent symbiosis and general permeation of the host by the bacteria is, of course, of con-

[1] K. F. Kellerman, U. S. Dept. Agr. *Yearbook*, 1910, p. 213; K. Shibata and M. Tahara, *Bot. Mag.*, Tokyo, vol. 31, 1917, p. 157.

[2] Cauda, *Nuov. giorn. botan.*, vol. 26, 1919, p. 169.

[3] Peklo, *Zeitschr. f. Gärungsphysiol.*, vol. 2, 1913, p. 275; M. Ch. Rayner, *Bot. Gaz.*, vol. 73, 1922, p. 226; E. Melin, *Jour. Ecol.* vol. 9, 1922, p. 254.

[4] B. M. Duggar and A. R. Davis, *Ann. Mo. Bot. Garden*, vol. 3, 1916, p. 413; Rayner, l. c.

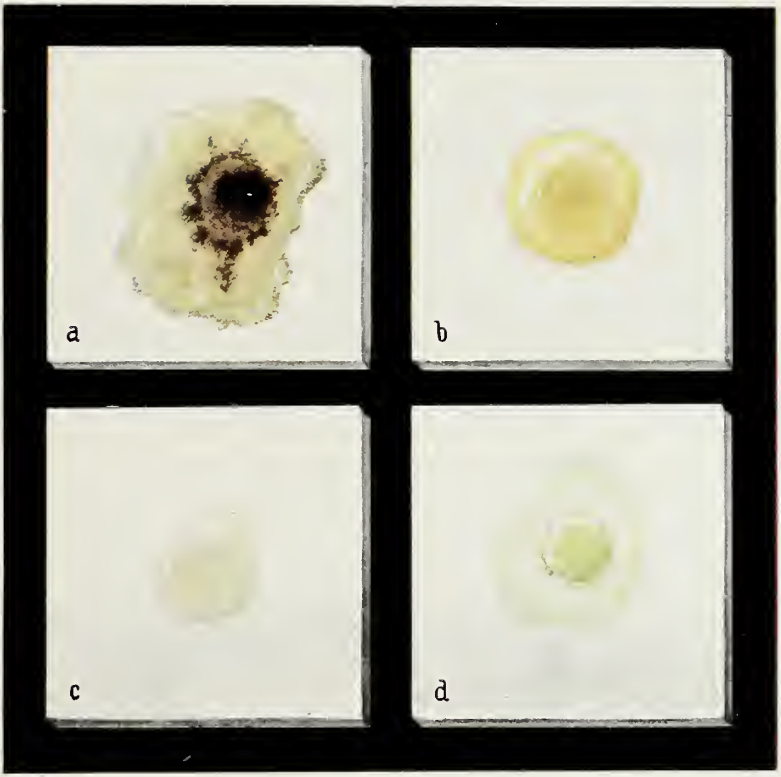
siderable advantage. Nitrogen fixation in pure cultures has been observed.[1]

Nitrogen Fixation in the Soil.—In addition to the nitrogen fixation caused by the nitrogen assimilating microorganisms living in symbiosis with higher plants, another mode of nitrogen fixation, less conspicuous but more general, takes place in every soil due to the activity of free living bacteria, fungi, and algae. As long as thinking men have cultivated the soil they have been aware that there is a general tendency in all soils to increase or to restore their productivity automatically. Barren rocks, which contain only minute traces of nitrogen compounds, undergo a slow process of disintegration and, if the climate permits, they transform themselves gradually into fertile soils. Lichens and mosses appear first, herbs and shrubs follow them, and if the annual precipitation suffices, ultimately trees get a foothold and develop to forests, which produce continuously enormous quantities of leaves and wood without any fertilization, provided that this great natural productivity is not destroyed by reckless forest devastation. Depressions in sterile sand which hold some water give rise to a growth of algae and mosses, which in turn preserve more water like a large sponge, and gradually grow up to extended deposits of peat. As mentioned above, nitrogen fixation takes place in certain herbs, shrubs, and trees growing in the woods and on peat land. Furthermore, nitrogen compounds are washed from the air into the soil by rain and snow. But the quantities of nitrogen brought down annually are not very large; according to many determinations made in all parts of the world an annual gain of 5-10 lbs. per acre may be expected. This is counterbalanced, however, by an annual loss in the drainage of about the same magnitude.[2]

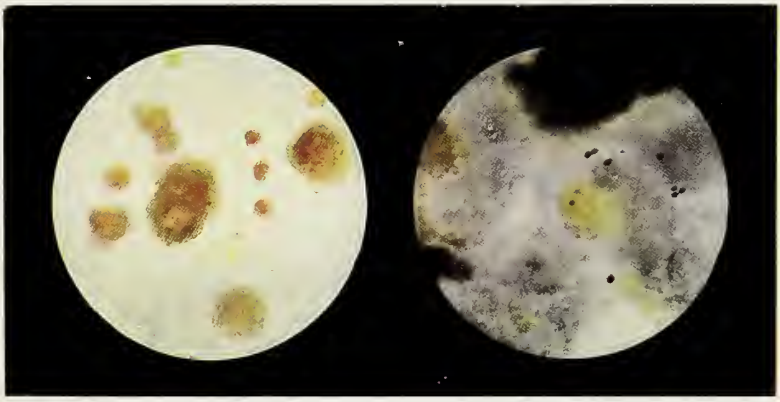
Many investigators have tried to discover the causes of this natural tendency of the soils to restore or to increase their fertility. The literature of the last century contains numerous chemical hypotheses which have been advanced to explain these gains in nitrogen by assuming that nitrogen fixation take place in soil under the influence of humus, or of iron and manganese oxides, or of ozone or of evaporating water, or of weak electric currents. But exact experiments have shown that another more potent cause must be active, and in 1885 it was discovered by the French chemist Marcellin Berthelot that this is the nitrogen assimilation performed by various microorganisms of the soil.

Nitrogen Assimilating Organisms in the Soil.—In 1893 the first results of investigations upon the nitrogen fixation in pure cultures of soil

[1] F. C. von Faber, *Jahrb. f. wissenschaftl. Botanik*, vol. 51, 1912, p. 285.
[2] Löhnis, "Handbuch der landw. Bakteriologie," 1910, pp. 635, 643.



1. Growth of Azotobacter on gypsum plates, nat. size
a. Azotobacter chroococcum b. Azotobacter Beijerinckii
c. Azotobacter vitreum d. Azotobacter agile



2. Aerobic cellulose decomposition, nat. size
Bacteria Fungi

bacteria were published by M. Berthelot, and a few weeks later additional data were furnished on the same subject by the Russian bacteriologist S. Winogradsky. Berthelot worked with different aerobic bacteria, while Winogradsky made his experiments with an anaerobic species which he called *Clostridium Pastorianum*. It was later proved by Bredemann that this separate name was not correctly applied. Winogradsky's organism is merely a variety of the common anaerobic butyric acid bacillus (*B. amylobacter*) which can be isolated from every soil, and which under suitable conditions always fixes some nitrogen. The gains recorded by Winogradsky (2 mg. N per 1 g. sugar) were no higher than those observed by Beijerinck and others in tests of *B. radicicola*. Later experiments showed gains up to 6 mg. per 1 g. sugar.

Still greater activity is usually displayed by *Azotobacter*, the large aerobic nitrogen fixing organism described by Beijerinck in 1901, which is shown in Fig. 5, Plate I. 10 to 15 mg. N per 1 g. sugar are frequently assimilated in routine experiments with these bacteria, but much larger gains can be obtained under more favorable conditions, especially if very young and vigorous cultures are tested.[1] Dextrose and mannitol are generally the best kinds of carbonaceous food, but numerous other carbohydrates, alcohols, and organic salts can be used. The products of the decomposition of cellulose and of pectic substances are equally accessible to Azotobacter; the symbiosis of such groups of organisms is undoubtedly of great benefit for the nitrogen fixation in soil. Various species or varieties of Azotobacter have been isolated; four of them are shown on Plate VIII. Beijerinck described two of them: *Azotobacter chroococcum*, characterized by its brown or black color, and *Azotobacter agile*, a rapidly motile form, producing in agar as well as in solution a brilliant green fluoreseence. *A. Vinelandii*, isolated by J. G. Lipman, is identical with *A. agile*. *A. Beijerinckii* is a variety of *A. chroococcum*, usually growing white but in certain stages of its development distinctly yellow. *A. vitreum* has never shown either pigmentation or motility, yet it seems to be a variety of *A. agile*. The pleomorphism of this group of organisms is very conspicuous; laboratory cultures often change from the large cell form to small coccoid and rod shaped types.[2]

During the last twenty years numerous other aerobic bacteria have been isolated from soils, which proved to be able to assimilate free nitrogen. Various cocci, non-sporulating and sporulating rods have been described. Several of them were recently found to be growth types of

[1] A. Koch und S. Seydel, *Centralbl. f. Bakt.*, II. Abt., vol. 31, 1911, p. 570.

[2] F. Löhnis and N. R. Smith, *Jour. Agric. Research*, vol. 6, 1916, p. 675; vol. 23, 1923, No. 6.

Azotobacter, therefore they are especially numerous in soils where the large cells of Azotobacter are temporarily scarce or absent. *Bact. lactis viscosum,* which causes ropiness in milk, is one of them; the sporulating *B. petasites* is another such form. *Bact. radiobacter,* which was mentioned above, is equally able to assimilate free nitrogen.

Green algae (Chlorophyceae) and blue green algae (Cyanophyceae) have also been tested repeatedly in regard to their abilities to fix nitrogen, in most cases with negative results. Nevertheless, they seem to participate actively in this process under natural conditions and further experiments may prove more enlightening. Small amounts of nitrates are useful in starting algal growth, and later the algae are able to assimilate free nitrogen.[1]

Nitrogen fixation in distinctly acid soils of high humus content seems to be due mainly to the presence of nitrogen assimilating fungi, but again most experiments thus far made with pure cultures have failed to furnish a satisfactory explanation. However, negative results should not be overrated, as is sometimes done. Experiments made with Azotobacter or Amylobacter do also not always show gains in nitrogen.

Importance of Nitrogen Fixation.—Despite the various doubtful points which are awaiting elucidation by future research, enough is known at present to secure much insight into the rôle played by nitrogen assimilating organisms in the soil. Because of the great number of species which are able to act in this manner, if conditions are favorable, it is evident that some nitrogen assimilation may be expected in every soil, as was indicated by practical experience. But the relation between carbon used and nitrogen fixed is always very wide; usually less, rarely more than 1 part of nitrogen is assimilated, while 100 parts of carbonaceous material are oxidized. Most soils, however, are not very rich in organic substances, and as far as these are available many microorganisms, which do not fix nitrogen, take their share, too. It is obvious that Azotobacter, Amylobacter, and their kind can never gather such large quantities of nitrogen in the soil, as can *B. radicicola* in the roots of the legumes. A steady stream of soluble carbohydrates is here furnished by the host plant almost exclusively for the use of its symbiont, and the continual removal of the products of nitrogen assimilation by the plant tends to keep the efficiency of the bacteria at its maximum. Numerous tests have shown that 100 to 200 lbs. of nitrogen can be gathered in a good crop of leguminous plants per acre, while in the soil itself only $^1/_5$ or $^1/_{10}$ of these quantities can be assimilated, even if the conditions are very favorable. Occasionally the meager supply of organic sub-

[1] F. B. Wann, *Science N. S.*, vol. 51, 1920, p. 247.

stances furnished by the soil may be supplemented to some extent by carbohydrates produced by soil algae, which may enter into symbiotic relations with Azotobacter and other nitrogen assimilating bacteria. Such cooperation has repeatedly been observed in water, as well as in semi-arid soils; but also in such cases the actual gains can not be very large. A glance at a field of alfalfa, soy beans, or clover demonstrates at once that very great quantities of organic substances must be available in order to secure a comparatively large amount of nitrogen. Additional data will be given in Chapter XIV, 2.

4. THE CYCLE OF CARBON, OXYGEN, AND HYDROGEN

The assimilation of carbon dioxide by green plants is the fundamental reaction that leads to the formation of all carbonaceous substances which

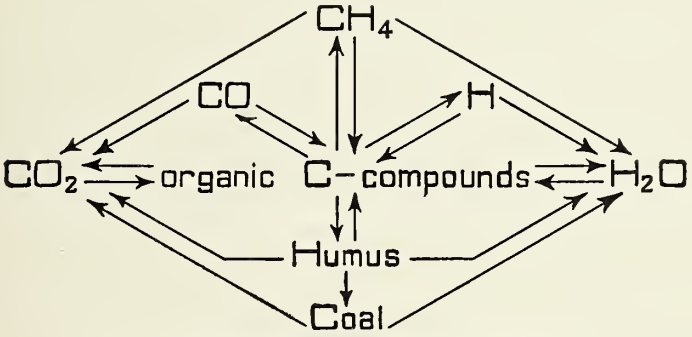


Fig. 30.—Cycle of carbon, oxygen and hydrogen.

participate in the construction of plants and animals. A few bacteria are able to perform a similar synthetic process, but the vast majority of microorganisms acts exclusively in the reverse direction. Organic compounds are oxidized by bacteria and fungi to carbon dioxide and water in a manner analogous to the respiratory process of the higher organisms. However, not all microorganisms live in the presence of air, and it is self-evident that under anaerobic conditions other reactions take place, leading to various intermediate compounds. But sooner or later these too will be mineralized by oxidizing microorganisms, so that the cycle of constructive and destructive processes will be completed.

Cycle of Carbon, Oxygen, and Hydrogen.—Figure 30 illustrates the different possibilities known at present. Besides the reactions taking place between the organic carbon compounds, carbon dioxide, and water, there are others by which several gases (carbon monoxide, methane, and hydrogen) or certain solid substances (humus and coal) are produced

as results of incomplete oxidation, and therefore subject to further transformation to carbon dioxide and water.

In regard to the organic carbon compounds it is the metabolism and final oxidation of carbohydrates, fats, alcohols, and organic acids which are of greatest practical importance. Benzol derivatives (phenols, etc.) may also be attacked by microorganisms, but these substances are generally much more resistant, and play a much less conspicuous rôle than the aliphatic compounds, as far as quantities are concerned. Because carbohydrates take part in the formation of organic nitrogenous compounds, they may, of course, appear as by-products in the course of bacterial destruction of proteins, or they may give rise to carbon dioxide and water.

What is called humus by the agriculturist is a complex and highly variable mixture of many organic as well as inorganic components. The chemical reactions taking place in the formation and destruction of humus in the soil are therefore not so well defined as they are with other organic substances. But because of the very prominent rôle they play in all soils, careful consideration will have to be given to them, too. As is indicated in Fig. 30, part of the humus may be used, especially by fungi, to produce well-defined organic substances within their own cells; the rest is slowly oxidized to carbon dioxide and water, or it is further reduced to coal-like material, such as is to be found in deep peat deposits of recent or ancient date. A gradual loss of oxygen and hydrogen together with a continual enrichment in carbon characterizes this process, which is, of course, not dependent on bacterial life. It has been repeatedly ascertained, however, that in the slow oxidation which is noticeable in stored coal bacteria may participate. But this process is of very little practical importance compared with the quick and nearly complete oxidation taking place in the burning of coal.

On the other hand, much of the carbon monoxide, methane, and hydrogen occurring in nature is not produced by bacteria, but is the result of volcanic activity and of incomplete combustion of coal. Numerous bacteria, however, participate in this process, and others, besides spontaneous oxidation, complete the transformations, making use of those gases for respiration as well as for assimilation.

Transformation of Sugar and of Starch.—The carbohydrates formed in green plants are used as far as possible for human and animal nutrition. This holds true especially in regard to sugar and starch, which, if they can not be used immediately, must always be protected against the attacks of microorganisms by heating, drying, or otherwise. In certain cases, however, a partial transformation of sugar and starch by bacterial activities proves useful and is therefore desired, for in-

stance in the ripening of cream and of cheese, in the preparation of silage, sauerkraut, etc. It is the anaerobic transformation of sugar into lactic acid which is of greatest importance in these cases; the acid produced acts as a preservative and at the same time increases the palatability of the food. In such cases varying quantities of other organic acids are also regularly formed. Formic, acetic, propionic, butyric, and succinic acids are most common. Acetic and butyric acids are sometimes very noticeable in spoiled silage, where either aerobic acetic and butyric acid bacteria may act, if the material is not properly packed and protected from the air, or anaerobic organisms may cause such deterioration, as they also do sometimes in faulty cheese. Before starch is acidified it is hydrolyzed to sugar by the amylolytic (or diastatic) action of various microorganisms. Many sporulating bacilli, as well as actinomycetes and molds, are active in this direction.

Lactic Acid Bacteria.—Several hundreds of so-called species of lactic acid bacteria have been described, capable of transforming the various sugars into either dextro, or laevo-lactic acid, or into the inactive modification. Smaller or larger quantities of by-products, mostly acetic and succinic acids, are formed, but this function can not be used for classifying the lactic acid bacteria, because it is too unstable and always influenced by the changing environmental conditions. All lactic acid bacteria of practical importance can be divided into the following four groups:

(1) Lactic acid streptococci (*Streptococcus lactis*);
(2) Lactobacilli (*Bacterium casei*);
(3) Intestinal lactic acid bacteria (*Bact. coli*, *B. aerogenes*, and *B. acidi lactici*);
(4) Lactic acid micrococci (*Micrococcus lactis acidi*).

Certain sporulating bacilli and vibrios, for instance the cholera vibrio, are also able to produce some lactic acid, but they are of no interest in this connection. The lactic acid streptococci and lactobacilli stand first in importance, and they were therefore separated by some authors as the "true" lactic acid bacteria from the two other groups, which were classed as "pseudo"-lactic acid bacteria. It is to be admitted that in regard to quantity, as well as to quality, generally the production of lactic acid is much more conspicuous in the two groups first mentioned. But representatives of the two other groups are also very active acid producers in the intestinal tract, as well as in milk and dairy produce. One type belonging to the third group, described by Hueppe as *B. acidi lactici*, was for a long time considered to be the most important lactic acid organism, and lactic acid micrococci are by no means rare in butter as well as in cheese. Because of their inclination to live within the diges-

tive tract many intestinal lactic acid bacteria display characters (strong gas formation and production of disagreeable flavors) which are detrimental to milk, butter and cheese. The lactic acid micrococci, on the other hand, are closely related to other non-acid producing cocci which possess strong proteolytic abilities. Frequently both functions, acid production and proteolysis, may be exerted by the same strain of micrococci according to circumstances.

The lactic acid *streptococci* do not always grow as typically globular cells, and for a long time they have been classed as rods. The famed British surgeon John Lister published the first accurate description of such an organism in 1878 and named it *Bacterium lactis*. Some of his

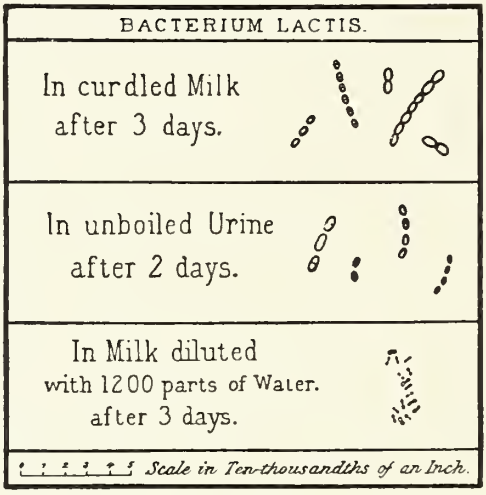


FIG. 31.—Bacterium lactis Lister, from "Transactions of the Pathological Society of London," Vol. XXIX, 1878, Plate XX.

drawings are reproduced in Fig. 31. The variability of the cell form is clearly demonstrated; other illustrations are given as Figs. 2 and 3 on Plate I. About twenty years later the same organism was described as *Bacterium lactis acidi* by Leichmann, and this name has been widely used in the dairy literature until quite recently. Many other names have been proposed, usually on account of rather unimportant differences of the strains studied. At present, however, it is almost generally admitted that this group of organisms should be classed as streptococci, and that the type species should bear the name *Streptococcus lactis*.

The *lactobacilli* grow mostly as slender rods of considerable length (Fig. 7, Plate I) and are much inclined to produce globular regenerative

bodies (Fig. 1, Plate II). While lactic acid streptococci predominate in sour milk, cream, butter, and in soft cheese, lactobacilli are usually more prevalent in silage and in hard cheese. Unfortunately, Leichmann named one of these forms *Bacillus lactis acidi,* as a counterpart to his *Bacterium lactis acidi* mentioned above. But because the names Bacillus and Bacterium are frequently used rather indiscriminately, much confusion has arisen and persisted for a long time in the literature. It seems best to use the name *Bacterium* or *Lactobacillus casei* as that of the typical representative of this group.

Naturally every strain of the lactic acid bacteria displays some features which frequently have been considered sufficient reason to create additional "new species," but a careful comparison of all these descriptions leaves no doubt that only the four groups mentioned are fairly well defined. Each of them can be subdivided, according to milk coagulation, gas formation, slime production, etc., into several types, wherein the several hundreds of so-called species of lactic acid bacteria find their places as more or less closely related varieties.[1]

It is noteworthy in this connection that the lactic acid streptococci as well as the micrococci are closely related to pathogenic species, namely *Streptococcus pyogenes* and *Micrococcus pyogenes,* and that the same holds true in regard to the intestinal lactic acid bacteria; *B. coli* being related to *B. typhosus,* and *B. aerogenes* to *B. pneumoniae.* These facts are of great importance especially as far as the microflora of the udder is concerned (see Chapter IX, 1).

Streptococci and lactobacilli generally grow best under anaerobic conditions, while the micrococci are distinctly aerobic. Certain varieties of the lactic acid bacteria act only upon sucrose (in silage), others only on lactose (in dairy produce), while many strains acidify these two sugars as well as others. But these behaviors are also subject to much variation.

Formation of Slime from Carbohydrates.—Milk, cream, bread, potatoes, and other substances rich in sugar or in starch become occasionally more or less slimy or ropy. The carbohydrates contained therein are consumed by microorganisms which make use of them for constructing large slime capsules around their cells, such as are shown in Fig. 10, Plate II. This peculiar behavior is not rare among the members of all four groups of lactic acid bacteria; especially the Streptococcus cultures used as starters for cream ripening display sometimes a marked

[1] Lõhnis, *Centralbl. f. Bakt.,* II. Abt., vol. 18, 1907, p. 97–149; "Handbuch der landw. Bakteriologie," 1910, p. 192–202; S. Orla-Jensen, "The Lactic Acid Bacteria," *Mem. Acad. Copenhagen, Natur. and Mathem. Sci. Cl.,* 8th Ser., vol. 5, No. 2, 1919.

tendency to degenerate in this manner. *Oidium lactis* is also known to give rise occasionally to slime producing varieties.

Slimy, slightly acid milk or cream are well liked in certain localities, for instance the so-called "taette" or "taettemjölk" (literally "tight milk") in Norway and Sweden, the "long whey" (that is, stringy whey) in Holland, and the "fiili" or "püma" prepared by Finnish settlers in Minnesota.[1] Usually however this alteration of milk and cream is distinctly disliked, although the food value is not much impaired. The slime producing varieties of lactic acid bacteria have received several specific names, of which the following ones are frequently used for members of the first, third and fourth group of the lactic acid bacteria, respectively: *Streptococcus hollandicus, Bacterium lactis viscosum,* and *Micrococcus pituitoparus.* The so-called Leuconostoc (Fig. 10, Plate II) which has played a rather disadvantageous rôle in sugar factories, is another slime producing Streptococcus variety. Its action can be suppressed easily by keeping the temperature of the liquids permanently at or above 60° C. In the dairy a thorough disinfection of all utensils is usually sufficient to eliminate the trouble. Sometimes, however, the water contains slime producing organisms, mostly *Bact. lactis viscosum* which is common in soil. Such water must be boiled, if no supply of pure water is available.

In starchy food, especially in bread, slime production is always due to the activity of certain sporulating bacilli (*B. mesentericus*) which may also become detrimental in sugar factories. Because of their great resistance, part of the spores survive the high temperatures in the baking oven, and they may afterwards cause a very disagreeable spoilage of such bread, if this is not kept at a low temperature. Addition of acid (sour milk or leavens) proves helpful, too, because the spores do not germinate in an acid substrate.

Formation of Alcohol.—The acidification of carbohydrates is always accompanied in nature by the formation of alcohol. Almost invariably a symbiosis exists between lactic acid bacteria, mostly lactobacilli, and ethyl-alcohol producing yeasts. Furthermore, several bacteria are able to produce acids as well as ethyl and other alcohols. Mannitol, for instance, is a by-product of the metabolism of many lactic acid bacteria. The quantities of alcohol may be small, as in sour milk, cream, and cheese, or moderate, as in sauerkraut and in silage, or large, as in the mash used in the fermentation industries. The various alcohols contribute, in combination with different acids, very materially to the flavors of dairy products and of silage. Certain oriental tribes prepare peculiar

[1] H. Macy, *Abstracts of Bacteriology,* vol. 6, 1922, p. 18.

fermented milks, such as kefir and koumiss, to which even curative qualities are ascribed, and which therefore are used to some extent in European as well as in American sanatoriums. Koumiss can be properly prepared only from mares' milk, which gives a specially flavored and much more alcoholic drink than can be obtained from cows' milk. Still stronger drinks are produced in Siberia by distilling the koumiss.[1]

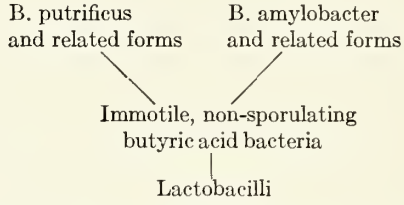
Transformation of Fats.—Fats in milk, cream, butter, and in cheese, as well as in concentrated feeding stuffs (rape cakes, etc.) may be more or less thoroughly transformed and destroyed by bacteria as well as by molds. As fats are compounds of organic acids (butyric, oleic, stearic, and other acids) with glycerol, an alcohol of high nutritive value, it is easily understood why many bacteria are inclined to split these substances, making use of the glycerol as a source of carbon, but leaving the free fatty acids which make those products more or less rancid. Oxidative processes may participate in the deterioration of the flavor of butter and cheese; sunlight and air themselves are able to exert such detrimental effects upon butter fat. As far as organisms are concerned they are almost exclusively aerobic bacteria and fungi. The latter do not make use of the glycerol only, but they destroy the fatty acids too, thereby producing considerable quantities of carbon dioxide and water, as was illustrated by the figures given on p. 45.

Butyric Acid Bacilli.—The peculiar flavor of rancid foodstuffs is due mostly to the presence of free butyric acid. This may be derived from fat; but there are numerous aerobic as well as anaerobic bacteria capable of producing butyric acid from carbohydrates. Diseased potatoes stored in pits are often destroyed by a rapidly progressing butyric acid fermentation which transforms their substance into an evil smelling viscous liquid. The most active butyric acid bacteria are anaerobic. As usual, numerous species have been created, but there is little doubt that most of them are in fact merely varieties of one species which has received several names; *Bacillus amylobacter* or *Clostridium butyricum* are the designations most commonly used. These bacilli produce rather large endospores which frequently, though not always, cause a swelling of the sporulating cell, so that it presents a club-like appearance (Fig. 12, Plate II). It is because of this peculiarity that the genus name Clostridium was introduced, but as this feature is by no means constant the name is not well founded. Variations are frequent within this group in two directions.[2] Either spore formation as well as motility vanish and the production of butyric acid is largely replaced by the for-

[1] B. Rubinsky, *Centralbl. f. Bakt.*, II. Abt., vol. 28, 1910, p. 161–219.

[2] Grassberger und Schattenfroh, *Archiv f. Hyg.*, vol. 60, 1907, pp. 40–78; G. Bredemann, *Centralbl. f. Bakt.*, II. Abt., vol. 23, 1909, pp 385–568.

mation of other acids, mostly lactic acid, or the spores appear in terminal position (so-called Plectridium type) and instead of acidification a proteolytic action becomes more and more noticeable. In the first case the relation to the lactobacilli becomes evident, while in the second case the general character is more or less similar to that of the anaerobic putrefying bacteria, *B. putrificus* and related forms. All these relations between the various groups of anaerobic bacteria can be illustrated in the following manner:



It is self-evident that profound changes in the general character can be observed only in experiments of long duration. Short termed investigations may easily lead to the erroneous conclusion that one or the other character is quite constant. On such basis, scores of species and dozens of genera of anaerobes have been proposed which are not tenable.

The aerobic butyric acid bacilli are related to the Amylobacter group as well as to the common hay and potato bacilli, *B. subtilis* and *B. mesentericus*. They play, as a rule, an inconspicuous rôle in nature.

Decomposition of Pectic Substances.—Wherever plant residues are decomposed in nature the dissolution of their pectic substances represents an important step, because these compounds participate largely in uniting the separate cells into solid tissues. If they are dissolved a general disintegration takes place, as is noticeable, for instance, in potatoes undergoing the butyric acid fermentation.

A particular type of disintegration takes place in the retting of flax, hemp, and other similar plants used in the textile industry. It is desired in these cases that in addition to sugar and starch the pectic substances be dissolved, but that the cellulose which forms the fibers, shall remain untouched. Chemical as well as biological methods are available for this purpose. The latter make use of either anaerobic or aerobic organisms, which in most cases are again closely related to the butyric acid bacteria just discussed. The forms active under anaerobic conditions are usually called *Plectridium* or *Granulobacter pectinovorum;* but it has been proved that they are varieties of *B. amylobacter.*[1] If the retting takes place in the presence of air certain sporulating bacilli,

[1] G. Bredemann, *Centralbl. f. Bakt.*, II. Abt., vol. 23, 1909, p. 385–568.

related to *B. subtilis* and *B. mesentericus*, may become active, or various molds may enter into the process; but these fungi are very likely to destroy simultaneously part of the cellulose and cause thereby a deterioration in the quality of the fibers.

Generally the most satisfactory results are obtained if the retting proceeds anaerobically under water and at a somewhat elevated temperature. Besides gases the bacteria produce various organic acids, which must be either neutralized or completely removed. In the latter case the water is constantly changed; in the former neutralizing substances, such as lime, soda, or potash, are added to the water.

Cellulose Decomposition.—The final step in the decomposition of plant tissues is represented by the destruction of the cellulose, generally the most resistant part of the vegetable cells. Again the process may occur under anaerobic or under aerobic conditions; usually it is more rapid in the latter case.

The organisms responsible for the anaerobic cellulose decomposition are not yet fully known. The problem has been studied by V. Omelianski of Petrograd, Russia, who described two types of slender bacilli with terminal spores, which, however, could not be grown in pure culture. One was characterized by its ability to produce methane in addition to carbon dioxide and various fatty acids, while the other evolved hydrogen.[1] Examinations of the original cultures, made by K. F. Kellerman and his collaborators,[2] did not confirm the findings of the Russian bacteriologist. Only aerobic cellulose bacteria could be found in Omelianski's cultures as well as in soil and manure. Therefore, new investigations will have to be made in order to explain these differences.

The first step in the decomposition of cellulose is its transformation into cellobiose and glucose. These sugars are quickly attacked by numerous bacteria which produce acids and gases, and it is very essential that these by-products be continually removed if a rapid cellulose decomposition under anaerobic conditions is to take place. Figure 32 demonstrates the different results obtainable in such experiments. In the intestines as well as in the manure pile the intermediate products are quickly destroyed by other microorganisms, provided that the reaction is alkaline. The bacterial processes are in both cases favored by relatively high temperatures. Even at 50° C. the decomposition of cellulose is fairly rapid, due to the participation of thermophilic organisms.

If the decomposition proceeds in the presence of air, as in soil, various non-sporulating as well as sporulating bacteria and also different

[1] Omelianski, *Centralbl. f. Bakt.*, II. Abt., vol. 8, 1902, p. 324; vol. 11, 1904, p. 369.

[2] Kellerman and McBeth, *Centralbl. f. Bakt.*, II. Abt., 34, 1912, p. 485; Same, Scales and N. R. Smith, l. c., vol. 39, 1913, p. 502.

fungi may become active. Figure 2, Plate VIII, shows the growth of such bacteria and fungi on paper. The fungi produce dark humus-like substances, whereas the bacteria dissolve the cellulose, as a rule, completely, and oxidize at the same time the intermediate products to carbon dioxide and water. If nitrate is present, denitrifying bacteria

FIG. 32.—Decomposition of cellulose in solution (left) repeatedly changed, or (right) not changed.

may act, making use of the oxygen of the nitrate for the oxidation of the cellulose, according to the formula:

$$(C_6H_{10}O_5)_x + 8\ KNO_2 = 4\ KHCO_3 + 2\ K_2CO_3 + 4\ N_2 + 3\ H_2O$$

Oxidation of Organic Acids.—The various organic acids present in animal or plant residues, or produced by bacteria in the course of the decomposition of carbohydrates, alcohols, and other carbonaceous organic

substances, especially if the oxidative processes are restricted by the absence of air, will undergo further oxidation in the presence of air under the influence of numerous fungi and bacteria. Free acids are attacked mostly by fungi, while the bacteria generally prefer the neutral salts. The following table gives a summary of results obtained in such experiments:[1]

Decomposition Positive (+) or Negative (−)	Formic Acid	Acetic Acid	Propionic Acid	Lactic Acid	Succinic Acid	Malic Acid	Tartaric Acid	Citric Acid
Bacterium fluorescens	+	+	+	+	+	+	+	+
Bacterium prodigiosum	+	−	−	−	+	+	−	+
Bacterium erythrogenes	−	−	−	−	−	+	+	+
Bacterium aerogenes	+	−	−	+	+	+	−	+
Bacterium acidi lactici	+	+	−	+	+	+	+	+
Bacterium coli	+	+	+	+	+	+	+	+
Bact. vulgare (Proteus)	+	−	−	−	−	+	+	+
Bacillus subtilis	−	−	−	+	−	+	−	+
Bacillus mesentericus	+	+	+	+	+	+	-	+
Oidium lactis	−	+	−	+	−	−	−	−

Even such a highly resistant acid as oxalic acid which accumulates in considerable quantities in certain plants, for instance in the leaves of sugar beets, can be oxidized by different bacteria.

Formation and Destruction of Humus.—The decomposition of organic residues gives rise not only to numerous chemically well defined substances, but also to relatively complex products of brown or black color, collectively called humus. Its presence is very conspicuous in barnyard manure as well as in soil, and practical experience has always indicated that there is a close relationship between the fertility of a given soil and its humus content. The colloidal nature of the humus compounds enables them to store vast quantities of plant food in such a manner that it is easily accessible to the soil organisms as well as to the roots of the cultivated plants. Nearly all nitrogen present in soil is there in the form of humus nitrogen; the gradual decomposition of the humus liberates part of this nitrogen as ammonia which is quickly nitrified. Considerable quantities of carbon dioxide are evolved simultaneously, which increase the solubility of the mineral constituents of the soil. Insofar as the carbon dioxide escapes from the soil, it adds to the supply of carbon dioxide in the air that is available for the green plants.

The dark color of the humus compounds is often accepted as indi-

[1] A. Maassen, *Arb. a. d. kais. Gesundheitsamt*, vol. 12, 1896, p. 340-411.

cating a high carbon content of these substances. However, only in certain cases, especially in peat, is the percentage of carbon distinctly higher than in carbohydrates. The humus of rich field soils, on the other hand, is usually relatively low in carbon, but comparatively high in nitrogen, as may be seen from the following data:[1]

Percentage	Carbon	Nitrogen
Peat humus	52–64	0.5–4
Field humus	30–56	3–9

The relation of N : C in green plants is 1 : 25-40, but only 1 : 10-15 in field humus. This difference shows that relatively more of the nitrogenous plant components participate in humus formation than do the carbonaceous substances. If the transformation takes place under anaerobic conditions, as in peat deposits, more carbon is retained, while in well aerated soils more carbon dioxide is liberated by oxidative processes. The rather resistant feces of many large and small animals make up a large part of the humus in soil, as they do in barnyard manure. But also ammonia as well as certain amino acids give dark humus-like compounds when combined with carbohydrates.[2] When so much ammonium carbonate or sulfate (plus chalk) is added to straw that the mixture contains 0.7 per cent N, a darkly colored material is formed within 8-12 weeks, which looks and acts very similar to barnyard manure, and no longer exerts such detrimental effects in soil as are characteristic of fresh straw.[3]

The chemistry of humus is only partly known. O. Schreiner and his associates were able to isolate from different soils a large number of aliphatic and cyclic compounds, which participate in humus formation.[4] When humus is purified as far as possible, a distinctly acid character is noticeable, and it is these humic acids that are partly responsible for the sour character of peaty soils. In fertile soil, neutralization takes place by combination with ammonia, lime, and other basic substances, and such neutral or slightly alkaline humus is much more easily oxidized by fungi and bacteria, as well as by purely chemical processes. The end

[1] F. Löhnis, "Handbuch der landw. Bakteriologie" 1910, p. 550-554.

[2] Maillard, "Genèse des matières protéiques et des matières humiques." Paris, 1913, p. 301-396.

[3] H. B. Hutchinson and E. H. Richards, *Jour. Min. Agr.*, Gr. Britain, vol. 28, 1921, p. 398–411.

[4] E. C. Lathrop, *Journ. Franklin Inst.*, vol. 183, 1917, p. 169; J. J. Skinner, l. c., vol. 186, 1918, p. 165.

products are always carbon dioxide and water, but part of the humic substances are again used for rebuilding the cells of the active microorganisms.

Formation and Assimilation of Carbon Dioxide.—The decomposition of humus furnishes most of the carbon dioxide needed by the green plants for assimilation. Burning of wood and coal, the respiration of men, animals, and plants, as well as volcanic activities produce also considerable quantities of carbon dioxide. But in view of the fact that from every acre of land approximately 5000-10,000 lbs. of organic matter are harvested annually, of which perhaps two-third remains upon or returns to the soil in the form of crop residues, green manures, and straw in stable manures, where they fall prey to humification and carbon dioxide formation, it becomes evident that these processes are of superior importance in regard to the continuous production of food for men and animals. Free air contains as a rule not more than 0.03 to 0.04 per cent carbon dioxide, while the air inclosed in fertile soil often contains 0.5 to 3.0, and occasionally up to 10 per cent or more. As there is a continual exchange of oxygen and carbon dioxide between atmosphere and soil, the carbon dioxide of the soil becomes accessible to the growing plants, whose leaves are turning their stomata, as a rule, toward the soil. There is no doubt that the greater supply of carbon dioxide furnished by a soil rich in neutral humus, is largely responsible for the better crops obtainable upon such soils.

Soil organisms may also assimilate some carbon dioxide. If a rich flora of algae is present, as in wet soils, the effect may become noticeable. Furthermore, there are a few groups of bacteria—the nitrifying, hydrogen oxidizing, certain sulfur and iron bacteria—that are also able to assimilate carbon dioxide. But these activities are practically quite irrelevant. The nitrifying bacteria, which are most active in soil, assimilate only 1 part of carbon while oxidizing 35 to 40 parts of nitrogen. In other words, if 70 to 80 lbs. of nitrogen are nitrified in one acre of land, not more than 2 lbs. of carbon are assimilated, which, of course, is of no importance whatever when compared with the humus present in the soil and with the quantities of carbon added in green and stable manures.

Formation and Metabolism of Carbon Monoxide, Methane, and Hydrogen.—Considerable quantities of *carbon monoxide* are liberated in the incomplete combustion of coal. Small amounts have also been found in stable manure, where they are probably produced by bacterial activity. Nothing definite is known in this respect. But several microorganisms have been isolated that are able to assimilate and to oxidize carbon monoxide, despite its being poisonous to all other organisms.

Most common among them is *Bac. oligocarbophilus,* first studied by Beijerinck.

Large quantities of *methane* are produced in the absence of air by numerous bacteria from very different substances, such as proteins, amino acids, carbohydrates, organic acids, and alcohols. Gases within the intestinal tract and in barnyard manure contain frequently not less than 50 per cent of methane. Soil covered by water shows the same gas formation, especially if it is rich in organic substances. In well aerated soils, however, very little methane or none is to be found. A so-called *Bac. methanicus* and several other species have proved themselves capable of making use of methane for assimilation and for respiration.

Hydrogen is produced under the same conditions as is methane, although usually in smaller quantities. Its oxidation occurs partly spontaneously, partly as a result of the activity of various bacteria that make use of the energy obtained for the assimilation of carbon dioxide. But this metabolism is by no means their only mode of life. If there is no hydrogen, as in most soils, they live, like other microorganisms, on organic food, and they can therefore not be properly classed as a separate, well defined group of "autotrophic" bacteria.

5. TRANSFORMATION OF MINERAL SUBSTANCES

Bacteria and fungi are responsible for most of the transformations of carbon and of nitrogen. In regard to the metabolism of the so-called mineral substances, such as potassium, calcium, iron, sulfur, and phosphorus, bacterial action is much less conspicuous, although by no means unimportant. In the course of the decomposition of organic residues these substances are attacked by the same microorganisms which break down the carbon and nitrogen compounds. Physical and chemical factors, on the other hand, are mostly responsible for the disintegration of rocks and for the gradual increase in solubility of the mineral soil constituents. Carbon dioxide as well as organic and inorganic acids are active in these respects, and since large quantities of them are produced by microorganisms, the latter are again of importance although in an indirect manner. Soluble minerals are used by bacteria and fungi as by higher organisms for cell construction. The phosphorus requirement of many bacteria is relatively large, when compared with that of cultivated plants.

Mineralization of Organic Residues.—The mineralization of organic residues remains always more or less incomplete. Part of the nitrogen and carbon is assimilated by the active microorganisms, and also a part of the phosphorus, potassium, calcium, iron, and sulfur. Rarely more than 50 per cent of the nitrogen of barnyard manure is nitrified in the

soil during the first three or four years. Similar relations prevail in regard to the mineral constituents of stable manure, although the increase in solubility is usually somewhat greater than in the case of nitrogen. The following data were obtained from such experiments concerning the percentage of nitrogen, phosphorus, and potassium recovered in the crops grown:

Percentage Recovered	Nitrogen	Phosphorus	Potassium
Field experiments, 2 years [1]	28–34	25–34	43–70
3 years [2]	32–51	30–41	?
4 years [3]	7–46	10–76	22–85
Pot experiments, 3 years [2]	54	103	?

Similar results have been obtained, but within a much shorter time, when in fish guano the phosphorus soluble in carbonated water was determined, before and after the material was fed to animals.[4] Originally not more than 18 per cent was soluble; in the feces the solubility was increased to 52 to 68 per cent, and after the excreta were kept for two months 62 to 73 per cent of the phosphorus had been transformed. That in certain cases the opposite reaction, that is, the assimilation of mineral compounds may become more or less conspicuous, is illustrated, for instance, by the fact that phosphates added to stable manure lost within a relatively short time 24 to 64 per cent of their soluble phosphorus.[5]

Metabolism of Phosphorous Compounds.—To the organic phosphorous compounds belong the nucleoproteids, the phytins, and the phosphatides (lecithin), of which the latter, as a rule, are more easily decomposed than the former. Inorganic phosphates are frequent in soil, but the humus contains also varying amounts of organic phosphorus. Plant residues (green manure, straw) are comparatively rich in organic phosphorus, especially phytin; animal residues (guano, bone meal) are characterized by a relatively high content of phosphates. Whether there is a conspicuous change from the inorganic to the organic form or vice versa, is largely dependent on the supply of bacterial nutrients, especially of carbohydrates. The general situation is similar to that existing in the mineralization or assimilation of nitrogen compounds. The presence of carbohydrates stimulates assimilation and checks mineralization; lack of

[1] W. Schneidewind, *Landw. Jahrb.*, vol. 39, Erg. Bd. III, 1910, pp. 62-74.
[2] B. Welbel, *Travaux du Labor. chimique de la Stat. agr. de Ploty*, 1908, pp. 58, 62.
[3] B. Schulze, *Arb. Deutsch. Landw. Gesellsch.*, 198, 1911, pp. 167, 170, 174.
[4] O. Kellner, *Landw. Vers. Stat.*, vol. 20, 1877, p. 433.
[5] W. E. Tottingham and C. Hoffmann, Wis. Agr. Exp. Stat. *Research Bull.* 29, 1912.

carbohydrates exerts the opposite influence. Humus furnishes food for the assimilating microorganisms and fixes at the same time part of the phosphorus, as well as the ammonia, by absorption. The detailed study of these processes meets with difficulties, because the sterilization of a soil changes its absorptive power in a varying degree, and a smaller or larger part of the phosphorus is usually so firmly fixed by absorption that its solubility is not very different from that of the phosphorus which was assimilated by soil organisms.

Bacterial Action upon Phosphates.—Some decades ago, when raw bone meal was used as fertilizer, it was often mixed with animal manures and kept for several weeks before being applied. The increased efficiency of such "fermented" material was thought to be due to a better solubility of the phosphates, while in fact the ammonification of organic nitrogenous compounds and the removal of fatty substances were undoubtedly of much greater influence. It goes without saying that in all cases where the alkalinity is increased by ammonification very little dissolution of the phosphate can be expected. A prompt reaction is possible only where acids are present or are formed which transform the triphosphates into di- and mono-phosphates. Raw rock phosphates are therefore of value in distinctly acid soils and in prairieland where an exceptionally high humus content assures a vigorous formation of organic acids and of large quantities of carbon dioxide. Composting of rock phosphates with sulfur and soil can also serve as a means of increasing the solubility of the phosphates, provided that the bacterial formation of sulfuric acid proceeds vigorously enough to effect a quick transformation of the phosphates.[1] As a rule, the direct application of acid phosphate will be preferable. Nitrification is practically without effect upon the solubility of the phosphates in soil. The quantity of nitrous and nitric acid formed is so small compared with the basic substances present in an average soil, that hardly any free acid will have an opportunity to attack the phosphates.[2] The action of carbon dioxide is undoubtedly of greater effect in all soils which contain enough humus.

Bacterial Action upon Carbonates and Silicates.—The transformation of alkali and calcium carbonates and silicates shows many analogies to the metabolism of phosphorus compounds. The indirect effects exerted by microorganisms are again of greater importance than their direct actions. Carbon dioxide, organic acids, nitric and sulfuric acids increase the solubility of the carbonates and silicates. As a rule carbon dioxide

[1] J. G. Lipman et al., *Soil Science*, vol. 5, 1918, pp. 243–250; vol. 11, 1921, pp. 87–92.

[2] J. W. Ames and T. E. Richmond, *Soil Science*, vol. 6, 1918, pp. 351–364; W. P. Kelley, *Jour. Agric. Research*, vol. 12, 1918, pp. 671–683.

takes the first rank. It is well known that feldspar, mica, and similar minerals are very resistant, and special experiments made with the intention to secure a quicker disintegration by bacterial activity did not furnish any remarkable results.[1] Other less resistant silicates of potassium, such as are present in the so-called green sands of New Jersey and Maryland, were made somewhat more soluble when they were composted with sulfur and manure,[2] but also in this case an economic advantage is hardly to be expected.

A few exceptions are known where acids produced by bacteria have proved able to perform a strong solvent action. One of them is the gradual disintegration of a mountain peak in Switzerland (the so-called Faulhorn in the Berner Oberland) which is caused by excessive nitrification, due to an exceptionally high nitrogen content of the rocks. A rapid deterioration of concrete structures has taken place in some cases when they were immersed in water rich in sulfuric acid or sulfates. Like-

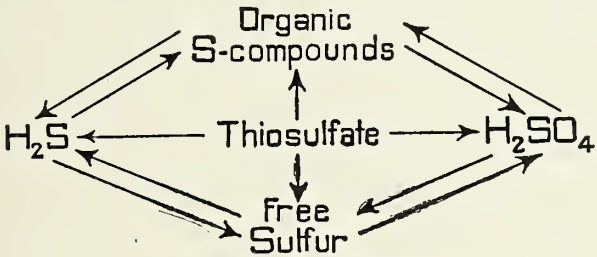


FIG. 33.—Sulfur cycle.

wise, organic acids have repeatedly caused serious damage by their destructive actions in dairies as well as in silos.

Sulfur Metabolism.—The transformation of sulfur and sulfur compounds shows many parallelisms to the cycle of nitrogen, as may be seen from a comparison of Fig. 33 with Fig. 29 (p. 96). In both cases the decomposition of the organic compounds leads at first to the formation of hydrogen compounds (NH_3 and SH_2), which are then oxidized to nitric and to sulfuric acid, respectively. A marked difference exists insofar as elementary sulfur, instead of nitrous acid, appears as an intermediate step in this process, according to the following formulae:

$$2\,H_2S+O_2=2\,H_2O+S_2$$

$$2\,H_2O+S_2+3\,O_2=2\,H_2SO_4$$

[1] BASSALIK, *Zeitschr. f. Gärungsphysiol.*, vol. 2, 1912, pp. 1–32; vol. 3, 1913, pp. 15–42.
[2] MCCALL and A. M. SMITH, *Jour. Agric. Research*, vol. 19, 1920, pp. 239–256.

Furthermore, it is to be pointed out that while sharply differentiated groups of microorganisms are active in the processes of ammonification and nitrification, the same bacteria are often able to produce hydrogen sulfide as well as sulfuric acid. In the absence of air the organic sulfur is mostly converted into hydrogen sulfide, but intensive aeration stimulates direct oxidation to sulfuric acid, which is immediately changed to sulfates.

The retrograde processes display also many parallelisms. Sulfates as well as free sulfur may be reduced to hydrogen sulfide; sulfates and hydrogen sulfide may be assimilated; free sulfur may be liberated from hydrogen sulfide as well as from sulfates. The thiosulfates which are produced by spontaneous oxidation of hydrogen sulfide and of other sulfides undergo analogous reactions. They may be reduced to hydrogen sulfide or oxidized to sulfates; at the same time elementary sulfur may be precipated and part of the sulfur may be used in the process of assimilation.

Purely chemical reactions play a much more important rôle in the transformation of sulfur and sulfur compounds than they do in the cycle of nitrogen. Hydrogen in the nascent state, as it occurs in many fermentative processes, may produce hydrogen sulfide from organic sulfur compounds, from elementary sulfur, from sulfites, or from thiosulfates. Sulfate reduction alone seems to be exclusively due to bacterial action, whereas the oxidation of hydrogen sulfide and of free sulfur to sulfates is again caused by mieroorganisms as well as by purely chemical reactions.

Formation of Hydrogen Sulfide.—Nearly all of the very numerous bacteria and fungi that are able to produce ammonia from organic substances have also been found to be connected with the production of hydrogen sulfide or of ammonium sulfide from proteins. This process occurs in the presence as well as in the absenee of air, at low as well as at high temperatures. Spoiled eggs are often, though not always, characterized by the liberated hydrogen sulfide or ammonium sulfide. The flavor of certain cheeses, such as Limburger, is partly due to hydrogen sulfide or ammonium sulfide; if traces of metal have entered such curd a gray or blaek discoloration of the cheese may become noticeable. Occasionally some sulfur may get into the milk from improperly vulcanized rubber tubes used on milking machines; the hydrogen sulfide produced causes a marked deterioration of the flavor of such milk, especially if this is kept at a relatively high temperature.

The offensive odors charaeteristic of putrid substances are probably always partly due to the presence of hydrogen sulfide and of ammonium sulfide, but volatile organic sulfur compounds, such as mercaptane, may contribute to the effect. Mercaptane is a by-product of the metabolism

1. Sulfur bacteria
$^2/_3$ nat. size

2. Iron bacteria
$^2/_3$ nat. size

of many baeteria that produee hydrogen sulfide; it is further split by them into aleohol and hydrogen sulfide.

Formation of Sulfates.—Organic sulfur eompounds, hydrogen sulfide, thiosulfates, and sulfur may all be oxidized to sulfates.[1] Abundant aeration induees many baeteria aeiive in the deeomposition of proteins, to transform the organic sulfur directly to sulfates instead of hydrogen sulfide. Hydrogen sulfide and other sulfides are equally attacked by numerous oxidizing bacteria, while thiosulfates and elementary sulfur are oxidized by only comparatively few species.

Compounds rich in oxygen, such as nitrates, can support these oxidations in the absenee of free oxygen, as illustrated in the formula:

$$12\ KNO_3 + 5\ S_2 + 4\ CaCO_3 = 6\ K_2SO_4 + 4\ CaSO_4 + 4\ CO_2 + 6\ N_2.$$

Denitrifieation takes plaee in this ease in the absence of organic substances; the active species has been named *Thiobacillus denitrificans.*

If mud rich in hydrogen sulfide, usually blackened by the sulfides which it contains, is kept under a layer of water of moderate depth, frequently the interesting phenomenon pictured in Plate IX becomes visible. Provided that the water in the cylinder is kept perfectly quiet and is protected from the light, the oxidizing bacteria will aeeumulate as a "plate" or "niveau" at a point in the solution where they get enough hydrogen sulfide from below and enough oxygen from above. The appendices visible below the small funnel-shaped depressions in the plate are formed by the motile baeteria diving down to gather the hydrogen sulfide and returning to the level where the oxidation is perfeeted. If the depth of the water were reduced the bacteria would return into the mud at the bottom, and the oxidation of the sulfides would become visible by a ehange in the color from blaek to gray.

Sulfur Bacteria.—Although all baeteria eonnected with the metabolism of sulfur are properly ealled sulfur bacteria, this designation is frequently used in a more restrieted sense for those baeteria only which oxidize hydrogen sulfide, sulfur, and thiosulfates to sulfates. The term thiosulfate bacteria is also applied to the last-named group of organisms, but they, too, are able to oxidize sulfur as well as hydrogen sulfide. As mentioned above, many species are known to be members of this group, and in regard to their morphology they display as many differences as have been observed with other baeteria.

[1] The term "sulfofication" is sometimes used to designate these processes, but it is as incorrectly chosen as were similar terms (azofication and rhizofication) mentioned on pp. 97 and 98. "Sulfofication" would mean formation, not oxidation, of sulfur.

Figure 34 shows a picture as it is often seen when a drop of water containing sulfur bacteria is examined under the microscope. Most of the cells visible are filled with minute droplets of colloidal sulfur, which is deposited within the cells as an intermediate product of the oxidation of hydrogen sulfide to sulfate. This accumulation of finely divided sulfur causes the whitish color of the plate of sulfur bacteria, as shown in Plate IX. Long threadlike forms (Beggiatoa, Thiothrix, and others) as well as short, mostly motile, rod-like and spiral cells are almost invariably to be found. If such mud water is exposed to the sun-light, a luxuriant growth of purple-colored bacteria frequently becomes established. These organisms are in part of very large dimensions and otherwise of peculiar appearance.[1]

FIG. 34.—Sulfur bacteria living. ×1000.

As causative agents of the oxidation of thiosulfates and of sulfur two species seem to be of greatest importance, that is, *Thiobacillus thioparus* and *Thiobacillus thiooxidans,* the latter being of special interest because of its inclination to grow in distinctly acid substrates and to produce and to withstand hydrogen-ion concentrations down to pH=0.6.[2] The oxidation of sulfur in phosphate-sulfur composts, mentioned on p. 132, is mainly due to its activity. When thiosulfates are oxidized, frequently part of the sulfur is temporarily deposited outside of, not within the cells, as in the case of Beggiatoa and other species oxidizing hydrogen sulfide. The reaction proceeds in the following manner:

$$2\,Na_2S_2O_3+O_2=2\,Na_2SO_4+S_2.$$

Part of the sulfur bacteria (Beggiatoa, Thiobacillus, and some others) make use of the energy liberated in the sulfate formation, for the assimilation of carbon dioxide, just as is done by the nitrifying bacteria in the oxidation of ammonia to nitrite and nitrate. Even the relations between nitrogen or sulfur oxidized and carbon assimilated are the same in both cases (40:1), as was ascertained in regard to sulfur oxidation by Thiobacillus.[3]

Sulfate Reduction.—The production of hydrogen sulfide from elementary sulfur is very common among bacteria and fungi, but the retrograde transformation of sulfates to hydrogen sulfide has thus far been

[1] H. MOLISCH, "Die Purpurbakterien," Jena, 1906.

[2] S. A. WAKSMAN and J. S. JOFFE, *Jour. Bact.*, vol. 7, 1922, pp. 239–256.

[3] LIESKE, *Sitzgs.-Ber. Akad. Heidelberg, Mathem.-naturw. Kl.*, (B) 1912, 6. Abhandlung.

observed with only two anaerobic spirilla. If gypsum is added to barnyard manure a very intensive reduction may become noticeable. Analogous changes may occur in ponds, water reservoirs, and also in waterlogged soils. Since the roots of most of the cultivated plants are very sensitive to hydrogen sulfide, proper aeration of field soils becomes of importance on this account, too.

Iron Metabolism.—Considerable quantities of iron are present in the form of carbonates or organic salts in the water circulating in the soil. To a lesser degree the same holds true in regard to manganese. By spontaneous oxidation of the bicarbonates the hydroxides of iron and manganese may be deposited, and part of the precipitates accumulating in water drains, conduits, ditches, as well as in the soil, are undoubtedly formed in this way.

However, numerous microorganisms are able to act in an analogous manner.[1] Clogging of water pipes by iron deposits has repeatedly been found to be due to the abundant growth of iron bacteria. These organisms make use either of the carbon dioxide of the carbonates, or of the organic acids of the iron and manganese salts. The first group represents another type of carbon dioxide assimilating microorganisms. The hydroxides of iron and manganese accumulate in smaller or larger quantities in colloidal form in the slimy capsules or sheaths of the iron bacteria. Later a slow crystallization takes place which destroys the soft bacterial cells and leaves the so-called bog ore, which presents in all respects the appearance of a material of purely mineral origin. Soils rich in humus may also contain an amount of hydroxides of iron and manganese held in suspension by the protective action of humus colloids. When the latter are destroyed by microorganisms, the hydroxides are precipitated upon the active cells.

Production of inorganic and of organic acids by bacteria and fungi may lead, on the other hand, to increased solubility of iron and manganese in the soil, and simultaneously to an increased iron content of the drainage water. Alfalfa and some other crops which leave large quantities of stubble and roots in the soil exert occasionally a very marked influence in this respect, because a more active decomposition takes place under such conditions.

Iron Bacteria.—Drainage water of peat soil is, as a rule, exceptionally rich in iron bacteria, which not only form brown deposits at the bottom of the ditches, but also accumulate in thin iridescent films on the surface of the water, where such accumulations have sometimes been mis-

[1] H. Molisch, "Die Eisenbakterien," 1910; E. C. Harder, U. S. Geological Survey, *Professional Paper* 113, 1919.

taken for oil. In Plate IX a cylinder is shown partly filled with swampy soil and water in which an iron rod has been immersed. As far as oxygen has permeated the water, the iron bacteria have settled upon the iron rod as well as on the side of the glass, and the characteristic film upon the surface has also been produced. Figure 35 demonstrates how these organisms appear when examined microscopically. Some of them are comparatively large; *Crenothrix, Gallionella,* and *Leptothrix* belong to this group. Numerous other species of average size and shape participate in these processes, as do even fungi and algae. As mentioned above, manganese may replace iron, and the oxidation of ferrous bicarbonate enables some of the iron bacteria to assimilate carbon dioxide.

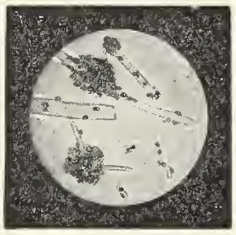


FIG. 35.—Iron bacteria living. ×1000.

6. PATHOGENIC ACTION OF MICROORGANISMS

Compared with the very large number of microorganisms continually active in nature as "mediators between death and life" of the higher organisms, there are only comparatively few species that are inclined to enter into close relations with higher plants and animals either on a symbiotic or on an antagonistic basis. The last-named group comprises all the pathogenic bacteria, whose intimate study represents the domain of medical bacteriology. Investigation and discussion of the infectious diseases of plants, animals, and men are the objects of plant pathology, and of veterinary and human medicine, as was pointed out on pp. 1 and 2. However, a general review of the activities of bacteria would be incomplete if the fundamental facts concerning the pathogenic action of microorganisms were entirely omitted. Therefore a brief discussion of these general principles will be given on the following pages.

Resistance Against Infectious Diseases.—As indicated by the term antagonistic action,[1] it is the struggle between the invading bacteria and the invaded organism that is characteristic of all infectious diseases. Either the bacteria are victorious and the higher organism succumbs, or the latter overcomes and kills the bacteria. This double-faced character of the problem is frequently overlooked. Especially during the first decades after the discovery of the causative agents of anthrax, cholera, typhoid, tuberculosis, etc., the bacteriological standpoint was often too much emphasized. Many people have lived and still live in permanent fear that they may come in contact with these dangerous bacteria. But

[1] Derived from the Greek word ἀνταγωνιστής (antagonistes) = opponent.

increased knowledge of bacterial action was accompanied by a growing insight into the various ways by which a healthy body offers a more or less successful resistance against infectious diseases. The hygienic point of view was thus brought to the foreground, and the belief was and is sometimes held that under proper hygienic conditions any action of the pathogenic bacteria is forestalled because of the resistance offered by a healthy body.

It need hardly be emphasized that both extreme views are not tenable. A large quantity of highly pathogenic bacteria introduced into a susceptible organism will promptly cause the disease, while this will not happen if only a few bacterial cells of reduced pathogenicity invade the host. The resistance offered by a healthy body is undoubtedly very great, but because no organism is continually in a state of perfect health, chances for falling sick are not infrequent. Therefore, the bacteriological and the hygienic points of view are of equal importance, although for practical reasons the latter may take first rank, as is expressed by the proverb: Prevention is better than cure.

Bacterial Pathogenicity.—In earlier times when pathogenic bacteria were still unknown there was a widespread belief that contagious diseases were closely related to putrefactive processes. This assumption was undoubtedly correct insofar as in such cases where proteins are decomposed by bacteria extremely poisonous substances—so-called ptomaines[1]—may be formed, which are capable of causing deadly diseases. Spoiled meat, cheese, and other decomposed foods rich in proteins may owe their more or less poisonous character to the presence of ptomaines. More frequently, however, a certain poison produced by a species called *B. botulinus* is responsible for food poisoning. Improperly treated silage as well as imperfectly sterilized food may contain this agent of botulism, which causes every year a considerable number of fatal accidents.

Other poisons, usually called toxins, are produced by the majority of pathogenic bacteria. In certain cases, however, the luxuriant growth of the microorganisms alone is sufficient to lead to serious disturbances in the host's metabolism, either by clogging part of the blood vessels or by otherwise interfering with the normal chemical processes.

The effects exerted by pathogenic bacteria are not always characterized by a very distinct, sharply defined and localized disease. Not infrequently different and variable symptoms may become noticeable and the pathogenicity itself may be more or less reduced. There are, in fact, many indications that the pathogenic bacteria are related to non-pathogenic forms, and it is quite probable that in the different stages of their

[1] Derived from the Greek word πτῶμα (ptoma) = corpse

development the pathogenicity, too, may vary widely. The sudden appearance and disappearance of epidemics, which is often very conspicuous, seems to have its main cause in these as yet little known features of the development of these organisms.[1]

Virulence and Infection.—Although bacterial pathogenicity is not invariably due to toxin production, it has nevertheless become a general usage to consider pathogenicity and virulence synonymous terms.[2] Strains are called highly virulent if their pathogenicity is at its height, and avirulent when they are no longer able to produce a disease. Avirulent strains are rather frequent with the relatively common infectious diseases, such as typhoid, diphtheria, and tuberculosis. But these so-called pseudo-diphtheria and pseudo-tuberculosis bacteria are also of great pathological interest, because they may regain their virulence under conditions not yet well known.

When pathogenic bacteria invade an organism it depends on their virulence and on the resistance offered by the invaded body whether or not an infection will take place. If a disease is caused, but the organism recuperates, the virulent bacteria may be retained in the body for a short, and sometimes for a long period. Such apparently healthy "carriers" of a disease are not uncommon, especially with typhoid, diphtheria, and cholera. Because the bacteria are still virulent in these cases, such carriers are liable to become a permanent danger to unprotected individuals.

The time which elapses until invasion is followed by infection is called the period of *incubation*.[3] Cell multiplication and toxin production takes place during this period until sufficient quantities of infective material are produced.

Endo- and Ecto-toxins.—Endo- and ecto-enzymes play prominent rôles in the metabolism of higher as well as of lower organisms. In the same manner the toxins produced by the bacterial cells are either retained therein as endo-toxins, or they leave the cells and act as so-called ecto-toxins. Generally the latter are more active than the former. The high mortality rate in fully developed cases of diphtheria and of tetanus is due to the presence of such ecto-toxins which quickly poison the whole organism. As in the case of the botulinus toxin mentioned above, heat, light, and various chemical substances can reduce or destroy the efficiency of these ecto-toxins.

The endo-toxins, obtained in the form of extracts from the cells by applying high pressure or by grinding the previously dried bacteria, do not act in such a specific manner as do the ecto-toxins. The living bac-

[1] ALMQUIST, *Jour. Infect. Diseases*, vol. 31, 1922, no. 5, p. 483.

[2] Virulence is derived from the Latin word virus = poison.

[3] Derived from the Latin word incubare = brood, hatch.

teria, however, act differently, and this may be due to their specific behavior within the body, as well as to the production of specific substances not yet well known. The so-called aggressins belong to this group; they have no marked toxic properties, but they stimulate the efficiency of the pathogenic bacteria very distinctly.

Immunity and Immunization.—If the resistance offered by an organism against a disease is sufficiently strong to destroy and to eliminate the pathogenic bacteria and to keep the body healthy, the organism is called immune against that kind of disease. If all individuals of the same species are immune against a disease, the immunity is absolute, but it is relative if only certain individuals prove to be resistant. Absolutely immune, for instance, are men against Rinderpest, cattle against glanders, most animals against typhoid. The relative immunity is strongly influenced by the age and the general living conditions of the individual; adverse factors (hunger, cold, fatigue) may impair the disease resistance seriously. The immunity may be inherited, or it may be later acquired either actively or passively, that is, either by having once been exposed to the disease, or by having been protected against it by direct immunization (application of immune serum or chemical treatment).

The reaction of the immune organism against bacterial infection must be twofold; the bacteria themselves must be killed and eliminated, and their toxins must be neutralized. If non-toxic bacteria invade an organism, as frequently happens with common non-pathogenic bacteria getting into lesions of the outer skin or of the inner lining of the body, the bactericidal action is very prompt. Because of the adverse environmental conditions the bacteria die within a short time and are speedily digested by enzymes of the blood. This prompt bactericidal action tends to keep the blood and inner organs of the animal body free from bacteria despite the frequent chances for contamination. If toxins are produced by the bacteria, the immune organism checks their effect by the production of so-called antitoxins, that are specific anti-bodies present in and transferable with the blood or the blood-serum of the immune organism.

Phagocytosis.—The fight against and the elimination of pathogenic bacteria is performed either by bacteriophagous cells or by specifically acting substances in the blood-serum (see bacteriolysis). The blood contains red corpuscles and white cells, so-called leucocytes. As was first observed by Metchnikoff, the latter are able to devour and to digest pathogenic bacteria, acting in this case as so-called *phagocytes*.[1] When examined microscopically such cells display a striking resemblance to certain protozoa (compare Fig. 36 with Fig. 16, Plate I, and Text-Fig.

[1] Derived from the Greek word φαγεῖν (phagein) = devour.

56). However, phagocytosis does not always proceed in a prompt and speedy manner. Sometimes the phagocytes seem to be paralyzed and unable to attack the bacteria. The bacterial aggressins mentioned above are accepted as cause of this inactivity, but their paralytic action can be overcome by an adequate counteraction of the infected body. Specific antiaggressins are produced by the blood-serum, which are usually called *opsonins*,[1] in order to indicate that they exert a stimulating effect upon the inclination of the phagocytes to ingest and to digest the aggressin producing bacteria.

Bacteriolysis and Agglutination.—If the blood of an immune organism is freed from its phagocytes and the serum alone is allowed to act upon the bacteria, again a bactericidal and bacteriolytic action may become noticeable, for which several substances have been made responsible. Some of them are produced by the phagocytes, while others, so-called

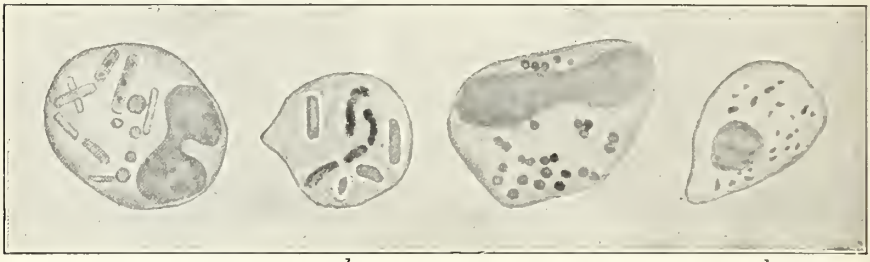


FIG. 36.—Phagocytes after Metchnikoff (*a–c*) and Bordet (*d*). *a–b*. Anthrax bacilli; *c*. Streptococci; *d*. Epithelioma contagiosum.

alexins,[2] are normal components of the immune serum. It was mentioned on p. 56 that those secreta of the leucocytes have been connected with the recently discovered bacteriophagy in the intestines (d'Herelle's phenomenon), although it is well beyond doubt that in this as in other cases where bacteriophagy was observed outside of the host, unknown substances or organisms are the cause of this antagonistic effect.

When bacteria are tested in blood-serum not always a genuine bacteriolysis takes place; the bacteria are not promptly killed and dissolved, but merely agglomerated and precipitated. This reaction, the so-called agglutination, is probably of no importance for the infected organism, but it is of considerable value for certain diagnostic purposes. In cases of typhoid, for instance, the *agglutination test* (Widal's reaction) has proved very helpful. Even the identification of non-pathogenic bacteria is frequently based upon the results of analogous tests. By inoculating

[1] Derived from the Greek word ὄψον (opson) = seasoning, relish.
[2] Derived from the Greek word ἀλέξειν (alexein) = ward off.

a certain strain of bacteria into the blood vessels of an animal, an immune serum can be obtained which agglutinates this as well as closely related strains of bacteria. But occasionally very erratic results are obtained.[1] For instance, serum of an animal immunized against tetanus agglutinated typhoid bacteria more vigorously than B. tetani; another serum expected to act specifically against mold spores, proved again more effective against typhoid bacteria. Therefore, too much stress should not be laid upon the outcome of such agglutination tests.

Hemolysis.—If instead of bacteria, blood of another organism is inoculated into an animal, again anti-bodies appear in the serum which exert in this case a lytic action upon the red corpuscles of the foreign blood. These hemolysins may be used to ascertain experimentally whether blood of unknown origin is that of man or of some kind of animal, and so to decide a question which in murder trials sometimes becomes of great importance. Analogous reactions are possible against many other substances, but of special interest is a peculiar test that is based upon the combined action of bacteriolysins and hemolysins in so-called inactivated and reactivated sera. The mechanism of this test is as follows:

Moderate heating makes a serum "inactive," that is, its bacteriolytic or hemolytic action is paralyzed; but it will be reactivated if a small amount of fresh serum is added. The substances which were destroyed by heating and are introduced again with the fresh serum are called complements, because the action of bacteriolysins and hemolysins will be complete only if these complements are present. If blood is mixed with inactivated hemolytic serum and bacteria are added in inactivated bacteriolytic serum, at first, of course, no reaction can take place in the mixture on account of the absence of complements. As soon as these are added, however, they will activate either the bacteriolysins or the hemolysins of the mixed sera; if the bacteria present are identical with those which were used for preparing the bacteriolytic serum, bacteriolysis will take place and no hemolysis, which becomes visible only if the bacteria are of another kind. This very valuable test has been invented by two Belgian bacteriologists, Bordet and Gengou; in its application upon the diagnosis of syphilis it has become known universally as the so-called Wassermann test. In doubtful cases of cholera, typhoid, etc., it can also be used to advantage.

Vaccination.—It was mentioned above that active immunization against an infection may have been acquired by previous exposure to the

[1] Lehmann und Neumann, "Grundriss der Bakteriologie," 5 Aufl., 1912, p. 135; Kruse, "Allgemeine Mikrobiologie," 1910, p. 1088.

same disease. In cases of measles, scarlet fever, whooping cough, for instance, the immunity acquired in early childhood is usually sufficient for the rest of the life. In other diseases, as in foot and mouth disease, the immunity obtained will persist only for a short period, and there are still other cases (influenza, gonorrhea) where practically no immunity against a new infection is secured. But wherever active immunization occurs, the possibility exists, as was discovered by Edward Jenner in regard to smallpox, that the immunization acquired from a relatively mild form of the disease will also afford protection against its more virulent forms. The inoculation of the vaccin taken from calves produces, as a rule, a mild local affliction which, however, creates an active immunity against the much more dangerous smallpox, and this immunity will last for a number of years. Since it is relatively easy to reduce experimentally the virulence of pure cultures of pathogenic bacteria to any desired degree, all kinds of vaccins can be prepared in the laboratories, and some of them have proved very helpful in combating infectious diseases. Anthrax, for instance, was formerly one of the most dreaded animal diseases, because the spores of *B. anthracis* remain alive in and on the soil for decades and are liable to cause new infections continually after one has occurred in a locality, but it has now lost much of its terror since Pasteur discovered a vaccination which has been much improved during the last thirty years.

Serum Treatment.—It takes time, of course, before an active immunization can be perfected by vaccination, and this is therefore of value only as a prophylactic treatment. But in certain cases it is possible to vaccinate experimental animals with gradually increased doses of bacteria or of toxins, until anti-bodies are formed in their blood in such large quantities that a comparatively small amount of the blood-serum carries enough protective substances to exert a prompt curative effect, if it is applied before the disease has been firmly established. This type of passive immunization has become of greatest importance and has proved highly beneficial in the treatment of diphtheria. Some other diseases are accessible to an analogous treatment, but the great hopes which followed Behring's discovery of the serum therapy of diphtheria were premature and exaggerated. The protection afforded by passive immunization can be completed by combining serum treatment with vaccination.

A peculiar fact that plays an important rôle in the preparation as well as in the application of immune serum is the so-called anaphylactic reaction of the treated organism. The term *anaphylaxis*[1] was chosen in

[1] Derived from the Greek words ἀνὰ (ana) = upward, and ἀφύλακτος (aphylaktos) = unguarded, defenceless; that is, anaphylaxis = increased defencelessness.

order to indicate that for some time after the first injection was made, an increased susceptibility is noticeable. If a first vaccination is quickly followed by a second injection, the anaphylactic shock produced may kill the experimental animal, but if this does not happen, anti-anaphylaxis is reached and further injections will do no harm.

Chemotherapy.—For a considerable length of time the interest of medical bacteriologists was concentrated almost exclusively upon vaccination and serum treatment. But it goes without saying that in the struggle against pathogenic bacteria the use of chemical substances may also be of great benefit. Mercury and arsenic compounds have proved very valuable in the treatment of syphilis, the application of chaulmoogra oil promises to eradicate leprosy, which has long been considered incurable. It is to be hoped that an equally effective treatment will be found against tuberculosis, since extensive experiments to produce active immunization by vaccination have not been successful. Other diseases, too, may be more accessible to chemotherapy than to vaccination and serum treatment. It depends on the circumstances which of the three methods will give the best results.

Part II

DAIRY AND SOIL BACTERIOLOGY

CHAPTER VIII

BACTERIA AND RELATED MICROORGANISMS IN FOODSTUFFS

As was pointed out in Chapter IV, 5, bacteria and related microorganisms accompany the cycle of matter that starts from and returns to the soil. Originally all microorganisms come from the soil, but the changes in environmental conditions cause numerous quantitative modifications in the microflora, as is found in foodstuffs, dairy produce, manure, etc. It was also mentioned before (p. 63) that all growing plants are covered by an almost continuous layer of bacteria specifically adapted to their habitat; in addition they are more or less contaminated by dirt, dust, and manure. The different ways in which foodstuffs are treated after having been harvested, lead necessarily to further alterations in the composition of the microflora in regard to number as well as kind of organisms present.

Germ Content of Foodstuffs.—When seeds are planted in sterilized soil rapid multiplication of the relatively few bacteria that stick to every seed starts as soon as germination takes place, and the young sprouts are being covered by the slimy layer of bacteria that is characteristic of all green plants. The following counts exemplify these relations:[1]

Number of bacteria	on each seed planted.....	3,000–80,000
	on young sprouts grown.....	750,000–19,750,000

Molds and bacteria may be found not only on the outside, but also in the inner parts of seeds. Leguminous seeds especially are sometimes heavily infested, as is indicated by their inability to develop normal sprouts in germination tests. In other cases the microorganisms within the seeds are of importance as symbionts, as was mentioned on p. 113.

The number of microorganisms present upon the different parts of full grown plants varies, of course, within wide limits according to plant species, conditions of growth, contaminations by dust, etc. The different

[1] Complete references relating to dairy and soil bacteriology are given in the senior author's "Handbuch der landwirtschaftlichen Bakteriologie," as far as they were published before autumn 1910. Additional summaries are printed in the *Zeitsch. f. Gärungsphysiologie*, vol. 1, 1912, pp. 68, 340, and in the *Centralbl. f. Bakt.*, II. Abt., vol. 54, 1921, p. 273.

treatment given to hay, silage, straw, and grain causes additional modifications. However, it is not so much the presence of microorganisms as it is their activities that are of real interest. It will suffice to give a few data showing the minimal and maximal counts of bacteria and fungi per gram.

Green forage	2,000,000–200,000,000
Hay	7,000,000– 17,000,000
Straw	10,000,000–400,000,000
Grain (cereals)	100,000– 12,000,000
Concentrated feed (oil cakes, etc.)	10,000– 20,000,000

Microflora of Different Foodstuffs.—Various organisms are growing in the slimy bacterial layer that is characteristic of the epidermis of green plants. Most common among them are *Bact. fluorescens* and a species called *Bact. herbicola;* the latter produces a yellow or reddish pigment that is sometimes visible with the naked eye on young sprouts, especially on fresh barley malt kept under humid conditions.[1] Plants which are supplied with stable manure are usually contaminated by fecal lactic acid bacteria (*B. coli* and *aerogenes*) ; if human feces were applied, pathogenic organisms, such as *B. typhosus,* may be found occasionally. Lactic acid streptococci and lactobacilli are, as a rule, comparatively rare on growing plants, but on cabbage and corn these organisms are more frequent, and these two plants are, therefore, especially inclined to undergo an acid fermentation in the sauerkraut vat or in the silo. Flax, hemp, and other fiber plants have also a peculiar microflora; bacteria and fungi attacking pectic substances are rather numerous in such cases. Spores of the common soil bacteria, such as *B. subtilis, mesentericus* and *amylobacter,* are carried by the dust as contaminations. Their presence and resistance make a complete sterilization of green vegetables sometimes difficult. Potatoes and beet-roots are, of course, exposed to very heavy contaminations by such organisms, whose growth may cause serious losses, if temperature and humidity are high in the places of storage. Still more accidental and irregular is the microflora of concentrated feeding stuffs. Some authors were of the opinion that bacteriological tests would allow of an accurate judgment in regard to the quality of such material, but the tests made did not confirm this hypothesis.

Grass, hay, and straw contain almost regularly, though not in great numbers, various representatives of a group of bacilli related to *B. tuberculosis.* Some of them have been explicitly named "grass bacilli" or "timothy bacilli." When found in milk, butter, and cheese, they have

[1] H. T. Güssow, Canada Expt. Farms *Report,* 1911, p. 241.

been repeatedly mistaken for true tubercle bacilli. In their typical form they are not pathogenic for men, but their virulence can be increased, and their general character may be so changed experimentally that they assume practically all the features of the tubercle bacillus.[1] However, under natural conditions this transformation will not often take place.

Hay Bacteria.—When grass is made into hay, part of the bacteria will die, but slime production and spore formation enable many of them to remain alive although in a dormant state. If the weather is favorable or the drying is done artificially, not many chemical alterations will take place. Under less favorable conditions, however, far-reaching changes may occur, and the nutritive value of the hay will be more or less impaired. As long as the water content of the material is not too low, the cell enzymes remain active, and the respiration of the cells goes on, resulting in a loss of approximately 10 per cent of the organic substances.[2] The specific aroma production is due to the splitting of certain glucosides. Part of the proteins (10 to 40 per cent) are transformed into amides and amino acids. The reduction in the percentage of carbohydrates, fats, and organic phosphorus compounds ranged, according to weather conditions, within the following limits:[3]

	Per Cent		Per Cent
Sucrose	22–87	Dextrin	0–45
Glucose	27–88	Fats	10–40
Starch	2–28	Phosphorus compounds	7–29

Phosphates increased accordingly, while cellulose as well as pectic substances remained unchanged as long as practically no bacterial activity took place. Unfavorable weather, however, stimulates unavoidably the growth of bacteria and molds, and their destructive activities become sometimes very marked, especially when clover or alfalfa is made into hay.

The so-called hay bacillus, *Bac. subtilis,* as well as other sporulating strains that are related to *Bac. mesentericus,* the so-called potato bacillus, can be easily brought to good development if hay is placed in water and the mixture boiled for a few minutes. After a few days the liquid is covered with a whitish film characteristic of these organisms.

Bacterial Activities in Stored Hay.—While hay making tends to suppress all bacterial activity, and this aim is, in fact, reached if the weather is not too unfavorable, bacteria will always become active again

[1] W. Kolle, H. Schlossberger und W. Pfannstiel, *Deutsche Medic. Wochenschr.*, vol. 47, 1921, p. 437.

[2] F. Fleischmann, *Landw. Vers. Stat.*, vol. 76, 1912, pp. 237–447.

[3] Fleischmann, l. c.

after the hay is stored, and the so-called sweating process sets in. The heat and moisture appearing in the hay stack at this time are in part the result of the respiration of surviving plant cells, but the increase in temperature and humidity favors the development of bacteria and fungi, which in turn add to the effect by their respiration. Under normal conditions all these activities keep within comparatively narrow limits. The rapid bacterial multiplication is followed by an equally sudden decrease in numbers, and after four to six weeks comparatively few vegetative cells together with more numerous spores may be found. Düggeli obtained, for instance, the following counts in millions per g. hay:

First Day	Seventh Day	Fourteenth Day
18	2,400	6

If the material was originally rich in *B. coli,* this species is especially liable to multiply rapidly, and together with some other bacteria it is probably the cause of the harmful effects repeatedly observed when such sweating hay was fed to horses before its "auto-sterilization" was complete.

The addition of 1 per cent salt proves helpful in suppressing excessive bacterial and mold growth in hay harvested in wet weather. If too much heat is generated in the hay stack, which may eventually lead to spontaneous ignition, as was discussed in Chapter VII, 1, brine or compressed carbon dioxide should be brought to the danger spots by means of gas pipes or similar appliances. The taking down of an endangered hay stack, which is often recommended, is highly dangerous because the access of air increases the chances for a sudden ignition. It should never be attempted unless plenty of water is available.

Sometimes hay is stacked while its water content is still comparatively high (approximately 45 per cent), and so-called *brown hay* is made. Enzymes of the plant cells and microorganisms combine their effects; 20 to 30 per cent of the organic substances are destroyed; proteins are reduced to amino acids and ammonia; and carbohydrates are changed to organic acids, alcohols, aldehyds, and carbon dioxide. This acid production together with the high temperature checks the bacterial activities, and a fairly valuable feeding stuff is obtained, although in most cases silage is superior, because here the unavoidable losses can be kept at a much lower level.

Making of Silage.—In Europe it has been known for centuries that it is possible to store plants rich in water and in carbohydrates in the absence of air without considerable losses, and that within a few weeks they are transformed into a palatable slightly acid product that keeps

well and represents a valuable winter forage. In the first decades of the 19th century many German and Hungarian farmers began to put part of their forage crops into pit silos, and in the sixties of the last century Reihlen of Württemberg started the ensiling of maize with very satisfactory results.[1] In 1870 his reports were translated into French, and from there the knowledge of this process came to America, where I. P. Roberts in New York, F. Morris in Maryland, M. Miles in Illinois and W. A. Henry in Wisconsin made the first silage. F. H. King also in Wisconsin carried out the first careful studies of the process and developed the American style of silo and ensiling. Recently the American system has been introduced into European farming, while the old European pit silos have come into use and have been improved upon in the Western part of America.[2]

At first it was thought necessary that the temperature in the ensiled fodder should rise to 50° C. in order to get silage of good quality, and this point is again frequently discussed in the more recent European literature. It was and is assumed that at these relatively high temperature a rapid development of favorable microorganisms takes place, and thereby the detrimental action of other bacteria is inhibited. This belief, however, is not well founded. The most important point is that by tight packing of the fodder the air be excluded as completely as possible. If this is done the temperature frequently does not exceed 30° C., and yet silage of perfect quality is obtained. Next to tight packing, the chemical composition of the ensiled material is of greatest importance. When the fodder is too young, that is, if it contains too much protein and water, a disagreeably smelling, unpalatable product will result. A relatively large percentage of carbohydrates must be present, and not more water than is needed to make juice enough to fill all air spaces between the fodder. Corn and sorghum are generally best suited for silage, because they are not too rich in proteins. Sunflowers, sweet clover, and a mixture of oats, vetch and peas or soy beans give also satisfactory results. It is much more difficult to turn clover and alfalfa into good silage; their moisture content is usually too high. The rapid formation of relatively large quantities of lactic acid is essential for securing good silage. If not enough lactic acid is present, butyric acid and disagreeably smelling products of the protein decomposition come to the foreground; such silage is of very inferior quality or a total loss. The following data[3] may indicate how the acid content of silage varies (percentage calculated on the dry weight basis):

[1] L. Carrier, *Jour. Amer. Soc. Agron.*, vol. 12, 1920, pp. 175–182.
[2] U. S. Dept. of Agr. *Farmers' Bull.* 825, 1917.
[3] R. E. Neidig, *Jour. Agric. Research*, vol. 14, 1918, pp. 395–409

Silage Made From	Acetic Acid	Propionic Acid	Butyric Acid	Lactic Acid
	Per Cent	Per Cent	Per Cent	Per Cent
Corn	1.8—4.0	0.2—0.3	0.0	4.0—6.1
Sunflowers	1.1—3.4	0.0—0.4	0.0—3.1	1.5—3.5
Oats and peas	1.5—2.2	0.1—0.2	0.0	4.6—5.0
Clover and straw	2.0—2.5	0.2	0.0	2.8—2.9
Alfalfa and straw	1.4—2.0	0.2—1.0	1.2—2.2	0.0

The making of good silage does not cause greater losses than approximately 10 per cent of the nutritive substances, which is no more than is lost when hay is made under favorable circumstances.

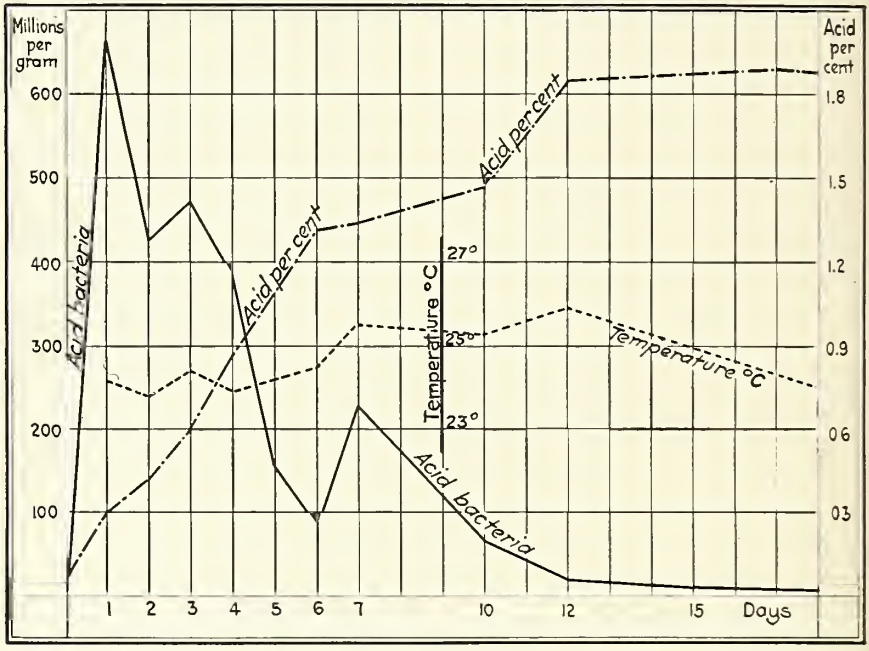


FIG. 37.—Acid-forming bacteria and acid production in corn silage, after Esten and Mason.[1]

Microflora of Silage.—Immediately after the fodder is put into the silo a rapid multiplication of bacteria, especially of acid producing species, takes place; 1000 millions per gram may be frequently found. But this rapid increase is followed by an equally rapid decrease, and

[1] ESTEN and MASON, Storrs Agr. Exper. Stat. *Bull.* 70., 1912.

after 2 to 4 weeks relatively few organisms are still alive; usually the lactobacilli are most persistent. The acid production proceeds more slowly than does the bacterial growth, as is shown in Fig. 37.

There has been much discussion in the literature whether the cell enzymes of the ensiled fodder or the microorganisms growing in the silage are of greater importance. It can not be doubted that the cell enzymes continue to work after the material is put into the silo, but it is equally certain that bacteria also play an important part in the formation of silage. Lactic acid bacteria, streptococci as well as lactobacilli, are numerous in good silage. Some of the latter reduce fructose to mannitol,[1] and it is due to their activity that silage is comparatively rich in this substance.[2] As usual, the lactobacilli are accompanied by yeasts; both groups of organisms transform part of the glucose to alcohol which is of influence upon the flavor of good silage. Part of the lactates first formed are later converted into acetates, and therefore the flavor of old silage is more pungent and sour. The lactobacilli themselves can participate in this secondary process; one of them, called Lactobacillus pentoaceticus, produced, for instance, in young cultures only 1 part acetic acid to every 8 parts of lactic acid, but in old cultures the relation was 1 : 2, because of the transformation of lactates.[3] Carbon dioxide and alcohol are likewise produced by lactobacilli as well as by cell enzymes. Occasionally carbon dioxide is evolved in such large quantities that it becomes dangerous to enter the silo, because of lack of oxygen for respiration.

Treatment of Silage.—If the material used for silage is naturally rich in carbohydrates, in plant enzymes, and in lactic acid bacteria, as is the case with corn, no other treatment is needed than the exclusion of air by tight packing. Material rich in protein must always be mixed with substances rich in carbohydrates, such as corn meal, straw, or molasses, and the addition of 1 per cent salt helps to suppress the protein decomposition without interfering with the acid formation. If there is a lack of active plant enzymes as well as of lactic acid bacteria, as is the case when silage is made from boiled potatoes, or from the waste products of beet sugar factories, the addition of active lactic acid bacteria is advisable and profitable. The use of such so-called pulp cultures originated in France and has spread to all European countries where the beet sugar industry furnishes much material for the pit silos. The photograph of one of the earliest pulp cultures, manufactured in Austria,

[1] E. B. Fred, W. H. Peterson and J. A. Anderson, *Jour. Biol. Chem.*, vol. 58, 1921, p. 385.

[2] Dox and Plaisance, *Science* 46, 1917, p. 192.

[3] W. H. Peterson and E. B. Fred, *Jour. Biol. Chem.*, vol. 42, 1920, p. 278.

is shown in Fig. 38. If such residues are not inoculated usually a vigorous formation of butyric acid takes place, because beets as well as beet tops are strongly contaminated by these common soil organisms whose spores survive the high temperatures applied in the sugar factories. Beet tops should always be kept as clean as possible. Heavily soiled material can never be made into good silage; putrefaction and butyric acid formation will predominate, and the silage produced will be either very inferior and dangerous, or a complete loss.

During the last few years an *electrical treatment* of silage has been

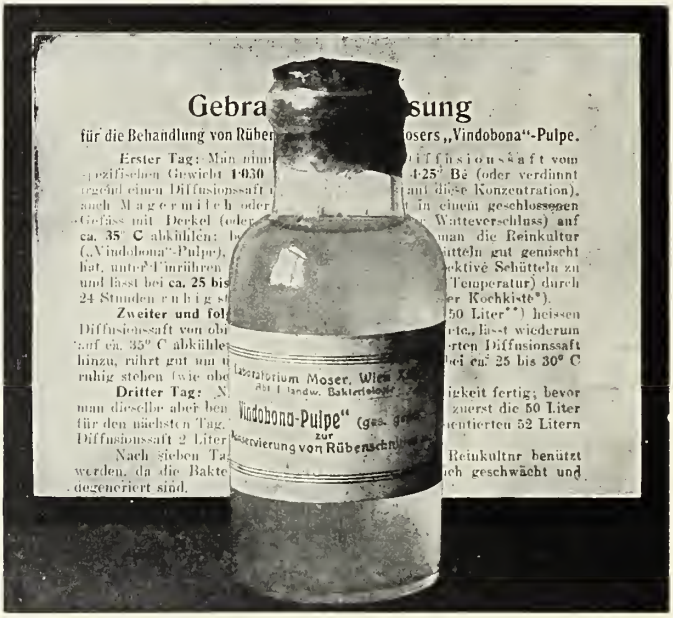


FIG. 38.—Pulp culture manufactured in Vienna, Austria, with directions (⅔ nat. size).

developed in Switzerland[1] that is now being tested extensively in different European countries. The results obtained are promising as far as the quality of the material is concerned. The passing of the electric current raises the temperature within the silo quickly to about 50° C., at which temperature only plant enzymes and certain lactobacilli display still enough activity to produce the necessary acidity. The fermentative processes, and therefore the losses of nutritive substances, are materially reduced, but it remains to be seen whether this advantage is great enough to justify the relatively high costs of the electrical treatment; about 1 kilowatt is required for every 100 lbs. of fodder. Several authors have

[1] TH. SCHWEIZER, *Deutsche landw. Presse*, vol. 48, 1921, p. 343.

asserted that all bacteria were killed by the electric current sent through the silage, but this statement is without foundation.

Sauerkraut.—The preservation of cabbage by spontaneous acid fermentation is practically the counterpart of the making of silage. It is very probable that the manufacture of sauerkraut, as practiced in Europe for centuries, has been the basis upon which all similar methods have been evolved. Cabbage, like corn, contains lactic acid bacteria which exert a very marked influence upon the quality of the product, although plant enzymes again participate in the process. The addition of salt to the cut cabbage, when it is being packed, acts favorably in two directions. It extracts by osmotic action the sap from the cells, which offers itself as a very suitable substrate for the lactic acid bacteria, and simultaneously keeps the air away from the submerged cabbage. Furthermore, many of the competitors of the lactic acid bacteria are checked, while the latter are little hindered by salt concentrations up to 3 per cent or more; and taste as well as flavor of the product is improved by the predominance of streptococci and lactobacilli. Yeasts, as the habitual symbionts of the lactobacilli, are regularly present in the sauerkraut vat and are partly responsible for the gas formation occuring therein. Occasionally pink yeasts may become so numerous that kraut of pinkish color and undesirable taste is produced.[1]

Other vegetables, such as cucumbers, green beans, etc., can be preserved in a similar manner, although with beans failures are not infrequent due to their higher protein content and a different microflora, wherein the lactic acid bacteria are not as predominant as they are on cabbage. Fresh juice from sauerkraut may be used for inoculation, while sour milk or whey are less suitable for this purpose; the lactic acid bacteria in the latter case being mostly adapted to milk sugar, not to the glucose present in vegetables.

Spoilage of Foodstuffs.—If the acid formation in silage remains low, the chances for spoilage are great, and it depends on the microflora accidentally present to what extent this will take place. Usually butyric acid formation and putrefactive processes will prevail, and the flavor of the material produced will be so disagreeable that the animals will refuse to eat it. Sometimes, however, it may happen that the fodder was contaminated by the spores of *B. botulinus*,[2] and such silage may become very dangerous, like the incompletely sterilized vegetables mentioned on p. 139, unless the germination of the botulinus spores is forestalled by prompt acidification.

[1] E. B. Fred and W. H. Peterson, *Jour. Bact.*, vol. 7, 1922, p. 257.

[2] J. S. Buckley and L. P. Shippen, *Jour. Amer. Vet. Assoc.*, vol. 50, 1917, p. 809.

Stored potatoes and beets are likewise exposed to the attacks of various bacilli and molds which may cause serious losses. If the tubers and roots are diseased when they are placed in storage, further deterioration can hardly be avoided. Healthy material, however, becomes subject to attacks only if parts of its tissues are first killed by high temperatures and excessive humidity, or by frost. Brown spots appearing in the white tissue of freshly cut potatoes indicate dead parts of the tubers. If such

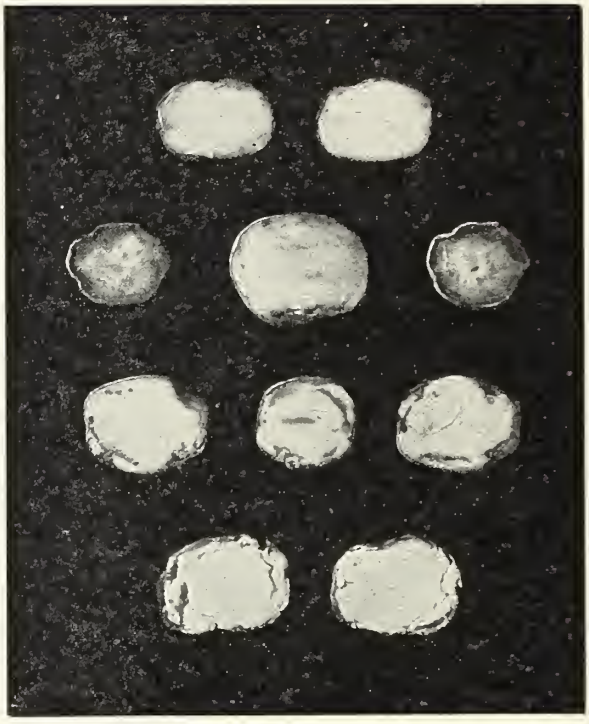


FIG. 39.—Cut potatoes showing the different types of decomposition ($\frac{1}{4}$ nat. size). Upper row: First appearance of brown spots. Second row: Change to dry rot. Third row: Change to bacterial rot. Lower row: Purely bacterial decomposition.

material is kept for some time exposed to the air, so-called dry rot sets in, that is, the tissue becomes a dry, powdery mass without the direct participation of bacteria; but bacteria become active whenever such potatoes are kept in an atmosphere that is saturated with humidity. Under these conditions anaerobic butyric acid bacteria become active; the pectic substances and part of the starch are decomposed, and a soft, ill-smelling material fills the skin. If the air was entirely excluded, not only the cells but the enzymes, too, are killed; the brownish discoloration is lacking in this case and the butyric acid bacteria alone are active. Figure 39 illus-

trates these various possibilities. The only way to avoid these alterations is by proper storage which prevents the death of the tissue.

Activities of Bacteria in the Digestive Tract.—As was discussed on p. 63, multiplication, decrease, and renewed multiplication of the microorganisms introduced with the food takes place in the different sections of the digestive tract, and certain changes in the composition of the microflora occur in accordance with the changes of environment. The rôle played by this intestinal flora has been extensively investigated. After many experimental difficulties had been overcome, it was repeatedly ascertained that it is possible to raise lower and higher animals under perfectly sterile conditions.[1] The presence of an intestinal microflora is therefore not absolutely necessary, but in different respects it is decidedly useful.

In the first place, bacteria may participate in the digestive processes. It is true that the animal organism produces numerous digestive enzymes and that they are of primary importance, but none of them seems to be able to attack the cellulose, which constitutes a relatively large percentage in the animal diet, especially in that of the ruminants. The activity of cellulose dissolving bacteria is of fundamental importance in this case. It is mainly due to their cooperation that the ruminants can make use of such large amounts of grass, hay, and straw, which ability makes them of such great economic importance. Furthermore, bacteria support and increase the effect of the digestive enzymes of the animal body in several directions, although these activities are less essential than is the dissolution of the cellulose.

In the second place, the products of the metabolism of the intestinal bacteria may exert a beneficial, but sometimes a detrimental influence upon the digestion, and thereby upon the general functioning of the higher organism. Again the activity of acid producing bacteria is of special advantage, because an acid reaction checks putrefactive processes, which otherwise may lead to the appearance of more or less toxic substances.

In the third place, the presence of the intestinal bacteria and of their metabolic products is decidedly useful whenever pathogenic bacteria may enter the animal body with the food. A few pathogenic microorganisms are soon overgrown by the many millions of intestinal bacteria, and the adaptation of the body to the various metabolic products of its intestinal flora causes a more or less marked immunity against bacterial toxins. This resistance was found to be entirely lacking in animals free of bacteria.

[1] E. Küster, *Arb. Kaiserl. Gesundh. Amt.*, vol. 48, 1914, p. 1; E. Wollman, *Bull. Inst. Pasteur*, vol. 12, 1914, p. 921.

Intestinal and Fecal Microflora.—In accordance with the conditions offered to the bacteria in the different parts of the digestive tract, a natural selection takes place among the species continually introduced with the food. Many of them succumb, while others are favored by their new environment and multiply rapidly. The result is that the latter form a rather constant intestinal flora, while the former represent a more accidental occurrence. Regularly present in large numbers are the intestinal lactic acid bacteria, that is, the motile *B. coli* and the immotile *B. aerogenes,* while the lactic acid streptococci and the lactobacilli are usually not very numerous, except when milk is fed, as to calves. Butyric acid bacilli and other anaerobic and aerobic sporulating species are very common, as are *B. fluorescens, proteus,* and related organisms.

The food consumed influences the intestinal and fecal microflora not so much by its own microflora as by its chemical qualities, which lead to more or less far-reaching changes in the composition of the intestinal flora, and may induce the development of varietal characters which often exert a very marked effect upon the milk and dairy products, if these are subject to fecal contaminations. For instance, if lactobacilli, such as are present in the intestine of milk fed animals, are added to other food, very few of them will survive in the digestive tract, but if milk or lactose is fed persistently, they will reappear and multiply enormously.

CHAPTER IX

BACTERIA AND RELATED MICROORGANISMS IN MILK

Milk and dairy products are of exceptional importance for human nutrition, and the monetary value of the quantities annually produced is very large. As is well known, bacterial growth and activity in milk may cause serious losses, and it was due to this fact that investigations upon the abnormal alterations of milk were part of the earliest work done in bacteriology (see pp. 8 and 89). Theoretically it would be best to protect milk from all contaminations, and to supply the consumer with a sterile product. Practically, however, the necessity of keeping the cost of milk production as low as possible, is usually the first item to be considered. The desire of the consumer to get milk at the lowest possible price, collides seriously with the request of hygienists to have the milk supplied free from undesirable microorganisms. To reconcile both points of view as far as possible, the milk producer needs an adequate knowledge of all pertinent facts, which have been thoroughly established during the last decades by dairy bacteriologists in all parts of the world.

1. GERM CONTENT OF MILK

The chances for contamination to which milk is exposed on its way from the cow's udder to the household, are, of course, very numerous. They begin in the udder itself and are dependent on the manner in which the milking is done and delivery to the consumer is made. According to circumstances, the various possibilities of contamination are to be investigated, and to be eliminated if this is feasible.

Bacteria Within the Udder.—As was mentioned before (p. 64), the cells of a healthy udder are free of bacteria, as is the blood, and the milk is sterile at the moment when it is formed. However, as soon as it passes down the ducts of the udder and accumulates within the cistern, contamination usually takes place. Occasionally, young healthy animals are found from which small samples of milk can be obtained free of all germs, but these are rather rare exceptions. As a rule, about 200 to 500 bacteria per cc. have been counted in milk from healthy cows when the milking was done aseptically and the vessels used had been sterilized before.

Only a few species of bacteria are regularly present in healthy udders, viz. certain strains of micrococci and of streptococci. A fat splitting variety of B. abortus seems also to be rather frequent,[1] although not so constant as the other two kinds. Other species, especially some causing abnormal alterations in milk, may appear temporarily, but they never become permanent inhabitants of the healthy cow's udder. The reason for this is that the healthy animal tissue possesses to a certain degree similar bactericidal properties as are characteristic of the healthy blood. Strictly non-pathogenic bacteria may invade the udder through the teats, but they are quickly suppressed and killed. The micrococci and streptococci first mentioned are also avirulent, as is the fat splitting variety of *B. abortus,* but all three are closely related to pathogenic forms (*Microc. pyogenes, Streptoc. pyogenes,* and *B. abortus,* the causative agent of infectious abortion of cattle), and therefore able to overcome to some extent the resistance offered by the healthy tissue.[2] It is a very peculiar adjustment and an accurately balanced equilibrium between these microorganisms and the animal body; they are not killed, but their multiplication is restricted, at least as long as the animal resistance is not weakened. If this occurs, however, as in cold rainy weather, or in sultry summer heat, or by blows against the udder, the chances are immediately offered for these bacteria to overcome the animal resistance, to multiply rapidly, to regain their suppressed virulence, and to cause what is commonly known as spontaneous inflammation (mastitis) of the udder.

These facts are of very great importance to the milk producer, as well as to the milk hygienist. Only when micrococci and streptococci are present in large numbers in the milk, is the suspicion justified that *inflammation of the udder* exists, is developing, or has been passed. If certified milk is being produced, such abnormal milk must be excluded, although it may be still serviceable for other purposes. If mastitis has fully developed, and especially if pus and blood are present, no further use of the milk is permissible. In regard to the udder variety of B. abortus, thus far no bad effects have been recorded in America, but in Mediterranean countries a disease called Malta fever, is widely spread by goat's milk that harbors a closely related organism.[3]

If *pathogenic organisms* circulate in the cow's blood, it is easily possible that they will appear in the milk, as is not infrequent with *B.*

[1] H. C. Cooledge, Michigan Agr. Exp. Stat. *Tech. Bull.* 33, 1916, and 41, 1918; A. C. Evans, *Jour. Infect. Diseases,* vol. 18, 1916, p. 437; vol. 23, 1918, p. 354; *Jour. Bact.,* vol. 2, 1917, p. 185.

[2] W. Steck, *Landw. Jahrb. d. Schweiz,* 1921, pp. 511–629.

[3] Z. Khaled, *Jour. Hyg.,* vol. 20, 1921, p. 319.

tuberculosis. It is equally possible that some of the pathogenic streptococci and micrococci may enter the udder with the blood. But in most cases the invasion comes from the outside, because the udder is always exposed to heavy contaminations from dust and feces. The small opening of the teat is wide enough to admit all bacteria, and the milk droplets often remaining there after milking permit a rapid multiplication of the invaders, which later advance upward into the cistern and the ducts of the udder. *B. coli,* the common fecal organism, is not as frequent in the udder as might be expected, but occasionally it appears in large numbers and may become the cause of another form of mastitis. This is also true in regard to *Bact. pyocyaneum.*

Fecal Contaminations.—The very high germ content of feces was pointed out before (p. 63), and it goes without saying that clean milk can not be obtained from unclean animals. Millions of bacteria are carried into the milk by every dirt particle loosened from the cow's udder or other parts of her body, and it is well worth knowing that even the inner parts of the teats of dirty udders may be so heavily infested with all kinds of bacteria that it will take weeks after the cow is thoroughly cleaned and groomed, before milk of low germ content is obtained. That the first few cubic centimeters of such milk must be very rich in germs needs no special explanation. The following examples[1] illustrate the general situation:

Bacteria per cc. Milk	First Part	Middle	End of Milking
From very clean animals.....	600	40	10
From moderately clean animals.	55,000–97,000	2,000–10,000	0–500
From dirty animals.....	6,500,000–86,000,000	?	12,000–43,000

If the udder is very dirty it should be washed with tepid water and soap, wiped dry with a soft towel, and the teats greased with some neutral fat (vaselin). If it is fairly clean, wiping with a dry soft cloth and light greasing is sufficient. Of course, no more vaselin should be used than is necessary to make the surface smooth and to fix the bacteria left firmly to the skin.[2]

Influence of Milking.—The persons who do the milking or are otherwise handling milk should be clean and healthy. Carriers of disease (see p. 140), especially those of typhoid and diphtheria, should never be

[1] More references are given in Chapter III, 1, of the "Handbuch der landwirtschaftlichen Bakteriologie."

[2] K. Volmer, "Ueber die beste Keimfreimachung des Euters." Diss. Bern., 1909, abstr. *Milchw. Zentralbl.*, vol. 7, p. 175.

allowed in such places. That from the dirty hands of a milker many organisms may enter the milk is self-evident, especially if the hands are kept wet while milking. If a man is not accustomed to milking with dry hands, he should grease them lightly, after they have been washed and dried. The milker's garment should be equally clean. Large washable coats or aprons are best; they may be sterilized, if certified milk is produced. It is to be highly recommended that the hands be washed again before another cow is milked, otherwise mastitis streptococci may be carried from diseased to healthy animals. The first milk, which is richest in germs and of low fat content, should be milked into a separate small container and discarded. It should not be milked on the ground, as is often done, because in this way mastitis bacteria find another opportunity of being carried from cow to cow.

That an unskilled and careless milker will cause greater contamination of the milk than a skilled and careful one, is beyond doubt; actual tests have shown that the difference may be ten-fold and more. *Covered pails* of simple construction, so that all parts of them are easily accessible to thorough cleaning, are far superior to open pails. The cover keeps many dirt and dust particles, hair, dandruff, etc. from falling into the milk. Accordingly, the germ content of milk in covered pails is usually found to be only 1/5 as high as that in open vessels.

Milking machines, unless constructed with care and kept scrupulously clean, increase the germ content of milk. Even comparatively minor details, such as imperfect check valves, may be the cause of heavy contaminations.[1] Thorough cleaning is insufficient, unless followed by a more or less complete sterilization of the whole apparatus. If a chemical treatment is considered, a combination of brine and hypochlorite has been found most satisfactory,[2] but very promising results have also been obtained by heating the carefully cleaned apparatus immersed in water in a covered boiler for 15 to 30 minutes at 75° to 85° C., and leaving it in the covered container, protected against all contaminations, until it is to be used again.[3] Theoretically the latter procedure is superior, but not all rubber stands the heat; in such cases the chemical treatment must be relied upon.[4]

Influence of Utensils.—From every container and every apparatus with which the milk comes into contact, from the milking pail to the de-

[1] R. S. Breed and J. W. Bright, New York State Agr. Exp. Stat. *Bull.* 488, 1921.

[2] G. L. A. Ruehle, R. S. Breed and G. A. Smith, New York State Agr. Exp. Stat. *Bull.* 450, 1918.

[3] G. H. Hart and W. H. Stabler, *Jour. Dairy Science,* vol. 3, 1920, pp. 33–51.

[4] R. S. Breed, *Jour. Dairy Science,* vol. 5, p. 102, 1922; A. H. Robertson, M. W. Finch and R. S. Breed, New York State Agr. Exp. Stat. *Bull.* 492, 1922.

livery bottle, a few or many bacteria are carried along, continually swelling the total germ content. The following figures may serve as an illustration:[1]

Germ Content of Milk	Per cc.
In the milking pail	19,000
In a second container	28,000
After passage of cooler	38,000
In a third container	78,000
After bottling	162,000

Cleanliness alone is again not a sufficient precaution if a low germ content is desired. Especially if cleansing soda is freely applied, it may happen that first of all the lactic acid bacteria are suppressed, while other species prove more resistant; the milk produced is then exposed to various unwelcome alterations, which are normally checked by the lactic acid fermentation. The cleaned and rinsed vessels should at least be *quickly dried,* so that the surviving bacteria can not multiply in the remaining droplets of water. This point is also of importance if the containers are *steamed,* because again not all bacteria and hardly any of their spores are killed, and these together with new contaminations may give rise to a very numerous and undesirable microflora. It has been ascertained repeatedly that such apparently clean, but in fact highly contaminated, containers may raise the germ content of the milk a hundred- or thousand-fold.

Thorough *sterilization* of pails, cans, and bottles is assured only if they are placed in a *hot air oven,* heated for a minute to 160° C. or for about 5 minutes to 140° C., and then kept in the closed apparatus until they are used. This system has been adopted by European milk producers during the last twenty years; recently it has also been recommended by American authors.[2]

Unfortunately, the *water* used for cleaning it not always as pure as it should be. Spores of butyric acid bacteria are by no means rare, *Bact. lactis viscosum,* which makes milk slimy, is rather common in impure water, and sometimes pathogenic bacteria may occur. Water of doubtful quality should be tested by adding some of it to clean milk; after one or a few days this milk is to be compared with another sample of the same milk to which no water had been added. If any suspicion arises as to the presence of pathogenic germs, thorough chlorination of the water is to be recommended.

Influence of Air and of Feeding.—As was pointed out above, cov-

[1] Backhaus und Cronheim, *Ber. d. landw. Instituts Königsberg,* vol. 2, 1898, p. 17.
[2] S. H. Ayers and C. S. Mudge, *Jour. Dairy Science,* vol. 4, 1921, p. 79.

ered milk pails are far superior to open ones, because they give to the milk sufficient protection against the "rain" of bacteria-laden dust, continually falling in the stable. That the milk should be transferred at once into another room where the air is not so infected, need hardly be emphasized, yet it is not always done. The high germ content of stable air can be easily demonstrated by exposing Petri dishes containing sterile gelatin for one minute, and by comparing the number of colonies growing on such plates with those obtained under analogous conditions in other rooms or in the open air.[1] If the litter is renewed, the manure removed, or dusty roughage is fed, the contamination of the air is much increased. Such manipulations should follow the milking, or they should be finished at least one hour before milking time so that the majority of bacteria and molds will have time to settle.

Contamination of the milk by the microflora of *food and water* may occur in three ways. Droplets of water and particles of food may be thrown into the air, or microorganisms from food and water may be transferred to the feces, or bacteria taken up through the mouth may enter the blood stream and get into the udder; but this last named possibility is restricted to pathogenic organisms. If the food acts unfavorably upon the digestion, fecal contaminations are unavoidable. If such material can not be entirely discarded, it should be mixed with other food that will eliminate its bad effect.

Most undesirable contaminations are caused by *flies*. A single fly may carry hundreds of millions of bacteria, and because these are mostly of fecal origin, and possibly of pathogenic character, a vigorous campaign against flies should be waged continually, wherever milk is exposed to the air.

Clarification of Milk.—Because of the facts discussed on pp. 40 and 64 it is impossible to attain a marked reduction of the germ content in milk by filtration or by the use of centrifugal power. Large clumps of bacteria and those adhering to dirt particles can be removed, but the majority will remain afloat. Many cell compounds are broken at the same time, and plate counts made before and after the milk has passed the filter or the centrifuge will, therefore, often show higher numbers in the clarified milk.

The desirability of removing dirt, hairs, dandruff, and other foreign material from milk necessitates, as a rule, one or the other method of clarification, which will be the more effective the earlier it is applied. Among the various filters those with a horizontal arrangement of cloth

[1] Photographs of such plates are given in F. Löhnis, "Laboratory Methods in Agricultural Bacteriology," Plate I.

or cotton are least desirable, because the dirt first deposited is washed and partially dissolved by the milk that follows. Only when a construction is chosen that forces the milk upward through the filter, is the horizontal position of the filtering surface not objectionable. Generally, however, funnel shaped filters are superior, because here the dirt can settle at the lower end without being further disturbed. Cotton is superior to filter cloth; if the latter is only washed, but not sterilized, it may become a source of heavy contaminations. A sample of milk poured through such material contained for example per cc.:

Before Filtration	After Filtration
20–420	140,000

Changes of Germ Content.—In view of the high nutritive value of milk it is to be expected that the bacteria which found their way into it, will multiply rapidly, especially if the milk is not cooled immediately after milking. If the initial germ content is high, it increases, indeed, very rapidly, but with clean milk the case is different, as many countings have shown. For instance, the following numbers were found per cc. milk:

Fresh	After 3 Hours	After 6 Hours	After 9 Hours
3090	920	1090	1160

It usually took 18 to 24 hours before the original germ content was restored, if this was low at the beginning.

This temporary reduction of the total number of bacteria present in milk is usually ascribed to *bactericidal* properties of fresh milk. Distinctly bactericidal actions are, in fact, clearly noticeable with colostrum milk, if this is kept at 37° C. Leucocytes (phagocytes) as well as the milk serum prove active in a similar, though not equally pronounced, manner to that characteristic of the blood. However, only part of the bacteria are really killed, while others are merely agglutinated and the number of their colonies growing on the plates therefore reduced. There are still other species which are not influenced at all; for instance, lactic acid streptococci begin their multiplication, as a rule, at once. If milk is heated for 20 to 30 minutes at 60° to 70° C. all bactericidal properties are destroyed, but reductions in germ content may still be observed. Not all bacteria find milk a suitable substrate, and they will die, of course, after a while, irrespective of any bactericidal action of the milk.

Influence of Keeping Milk at Different Temperatures.—Generally the multiplication of the bacteria, that always follows the temporary reduction, will be the more delayed the more the temperature of the milk

is reduced by cooling. For instance, milk with an original content of 5000 per cc. showed the following numbers per cc. after 24 hours:

At 5° C.	At 10° C.	At 18° C.	At 35° C.
2400	7000	280,000	12,500,000

Because lactic acid bacteria multiply very little at or below 10° C., this temperature (50° F.) is usually adopted as sufficiently low and yet not too difficult to reach and to maintain under practical conditions. It would be much more expensive to lower the temperature to 5° C. or less, and the effect would not justify the increase in costs, because between 0° and 5° C. different psychrophilic bacteria will multiply, among them B. fluorescens and certain micrococci, which attack casein and fat and cause a distinct deterioration of taste and flavor. The following counts were obtained per cc., when samples of fresh and of pasteurized milk were kept at 4° to 5° C.:

Counts per cc.	1 Day	2 Days	3 Days	4 Days	5 Days	6 Days
Fresh milk	21,120	23,680	121,080	338,560	innumerable	
Pasteurized milk	60	40	30	360	32,040	209,920

Curves showing the multiplication of some of the most common milk bacteria (Streptoc. lactis, B. coli, and fluorescens), when grown separately and in symbiosis at different temperatures, were presented in Fig. 17 (p. 57).

Influence of Transporting and Marketing Milk.—It is not too difficult to produce milk of low germ content, if all cows are healthy and, so far as practically possible, all sources of contamination are carefully avoided. 5000 to 10,000 bacteria per cc. of certified milk, and 50,000 to 100,000 per cc. of ordinary milk can be accepted as maximum at the places of milk production, but when such milk is tested at its place of destination, often one or several million bacteria per cc. are found, due to improper handling in transit. Use of unclean vessels for transportation and lack of protection against high temperature of the air are the two main factors which frequently nullify all efforts of careful milk producers. If the transport vessels are sent back to the producer without being thoroughly cleaned and sterilized, all kinds of undesirable bacteria have excellent opportunities of rapid multiplication, and every cubic centimeter of clean milk filled into such vessels is exposed to very serious contaminations.

In the second place, insufficient protection against high outside tem-

perature may accelerate the multiplication of the milk bacteria to such an extent that many millions per cc. may be present when the milk reaches its destination. General use of refrigerator cars for the transportation of milk is very desirable, and the stipulation contained in milk ordinances that milk should always be kept at a temperature not higher than 50° F. is very appropriate and useful, if properly enforced.

Carelessness in handling the milk in the household frequently adds to the bad effect of long unprotected transportation in hot weather, and the lower the original germ content has been, the larger will be its relative increase under such unsuitable conditions. The number of bacteria present at the time of delivery will increase about 100- to 200-fold when the milk is kept from morning until evening at summer temperature outside of the refrigerator. Compared with this last accumulative effect, all previous steps are of comparatively little importance. Certified milk with originally 5000 bacteria per cc. may ultimately harbor not less than 2,000,000, and ordinary milk which left the farm with a germ content of 100,000 per cc., will show not less than 10 millions, if handled in this faulty manner.

Germ Content of Different Kinds of Milk.—The establishment of three classes of milk—certified milk with less than 10,000 bacteria per cc., Grade A milk with less than 100,000 bacteria per cc., and pasteurized milk—is probably the relatively best arrangement which can be made. In several parts of the United States such or similar regulations are in force, while in other districts, and especially in European countries, many different arrangements have been adopted. It is, of course, next to impossible to harmonize the points of view held by milk producer, milk dealer, milk consumer, milk hygienist, and milk chemist. Raw milk of very low germ content and absolutely free of all pathogenic or otherwise harmful bacteria, that is, *certified milk,* is needed only in those cases where children do not thrive on pasteurized milk. The costs of production and handling are necessarily high, but if they save a child's life, they are well spent.[1] Milk of moderate germ content, that is below 100,000 or 200,000 per cc., represents an excellent *table milk,* provided that care is taken to exclude pathogenic bacteria, as well as milk of abnormal taste and flavor. Its production is less costly than that of certified milk, but sterilization of the utensils and careful cooling of the milk are necessary also in this case, and must be paid for by the consumer.

The higher germ content of all other milk may be reduced by pasteurization, which is relatively the cheapest method of rendering infected

[1] For details in regard to production and use of certified milk see *Proceedings* of the Annual Conferences of the American Association of Medical Milk Commissions.

milk harmless. However, one point is to be strongly emphasized concerning the general use of *pasteurized milk.* It is not very difficult to kill practically all bacteria that have grown in the milk, but their bodies, as well as their metabolic products, are not removed, and if the milk is not quickly cooled and always kept below 50° F. after pasteurization, the few surviving bacteria will multiply very rapidly and may cause alterations in the milk that will make it a dangerous food, especially for little children.

Simple determinations of the germ content of milk are of restricted value, and the results obtained in this way should not be overestimated. This holds true especially insofar as the *scoring* of dairy farms and dairies is concerned. Some authors are inclined to use the bacteria counts as a means to prove the inaccuracies of the various scoring systems.[1] However, if both determinations are properly made and the investigations are placed upon a broad basis, a fairly close parallelism is to be expected and has been actually observed.[2]

The situation is similar in regard to the relations existing between *dirt content* and germ content of milk. That there is no close parallelism between the two findings in individual cases has been shown by European investigators 20 or 30 years ago, and it is practically self-evident. Even if milk is handled in a rather unclean manner it will rarely contain more than 10 mg. of dirt, that is cow's feces, in 1000 cc. According to the data presented on p. 63, approximately 150 million of bacteria would be brought into the milk, or 150,000 per c.c., that is less than is often added by a contaminated container. Furthermore, visible dirt can be easily removed by filtration or centrifugation, while the germ content of such milk is not lowered thereby. If, however, filter tests are regularly made with many milk samples that have not been previously strained, it becomes obvious that the determination of the dirt content gives a fairly reliable indication of the bacterial quality of these milk samples, not only in regard to the number, but also concerning the kinds of bacteria present. Next to the presence of pathogenic organisms a heavy fecal contamination is, of course, least desirable.

Harmless and Pathogenic Bacteria in Milk.—One or several millions of bacteria occupy very little space in the milk, as was illustrated on pp. 18 and 64, and it goes without saying that ordinary milk containing 10 or 100 millions of lactic acid bacteria is far superior to milk with a low total germ content, but not absolutely free from pathogenic organisms. Tuberculosis, typhoid, scarlet fever, and diphtheria are occasion-

[1] J. A. Harris, *Science*, vol. 42, 1915, p. 503; Ch. E. North, *Amer. Jour. Publ. Health*, vol. 7, 1917, p. 25.

[2] I. V. Hiscock, *Jour. Dairy Science*, vol. 5, 1922, p. 83.

ally spread by milk, and if the udder harbors pathogenic streptococci, or the cows are infected with foot and mouth disease, such milk may also cause diseases in the human mouth, throat, and digestive tract. Many special tests have been invented to detect the presence of harmful bacteria in the milk, but the results obtained are not very satisfactory. Animal tests are necessary, for instance, to prove the absence or presence of tubercle bacilli in milk, and it takes weeks before a conclusion can be

Fig. 40.

Fig. 41.

Fig. 40.—Centrifuge for testing milk ($\frac{1}{6}$ nat. size).
Fig. 41.—Milk glass for mastitis test ($\frac{2}{3}$ nat. size.)

reached. Milk-borne infections of typhoid, scarlet fever, and of diphtheria can be traced only by careful examinations made by hygienists. This is the reason why pasteurization or boiling of the milk is to be recommended in all cases where the hygienic quality of the milk supply is not known.

Mastitis or Leucocyte Test.—The detection of a contamination by streptococci from an inflamed udder is comparatively easy. If 10 cc. samples of the milk are centrifuged in an apparatus like that shown in Figs. 40 and 41, and in the tapering end of the test glass a yellowish precipitate is thrown down which comes close to or surpasses the lines marked 1 and 2, equal to 1 or 2 parts per 1000, a microscopic examination of the sediment is to be made. If at 1000-fold magnification a picture is obtained similar to that in Fig. 42, the suspicion is justified that the milk contains secretions from a diseased udder. Presence of blood in the precipitate increases this probability.

Fig. 42.—Sediment of mastitis milk, stained. ×1000.

However, no final decision can be based upon this preliminary test. Milk drawn from healthy udders may sometimes contain rather large quantities of leucocytes, which form a heavy yellowish precipitate when the milk is centrifuged, and streptococci from other sources may grow in the milk and have the appearance of mastitis streptococci. Therefore, the examination must be extended to the place from which the milk was obtained, and it must be ascertained by individual tests whether some of the milk produced contains leucocytes (pus cells) as well as numerous streptococci. If this is the case, a clinical examination will almost invariably show that the udder of the particular animal is inflamed.

Counting Bacteria in Milk.—For a long time plate counts only were made for determining the total germ content of milk, but more recently microscopic examinations are made in increasing numbers. The latter are more reliable, especially if their results are compared with those obtained on thickly sown plates of beef agar kept only for a day or two. The necessity to get the results as early as possible, increases the usefulness of microscopic tests, while on the other hand their value is impaired by the fact that accurate counts are not secured if the total germ content is relatively low, as in Grade A and certified milks.

A quick and fairly accurate determination of the total germ content is, of course, very desirable especially in these two cases, and such a test has become possible by a combination of plate and microscopic examination is the so-called *little plate method*.[1] $^1/_{10}$ or $^1/_{20}$ cc. of milk is mixed with agar, and the mixture is spread on a measured area of a sterilized slide, which is kept at 38° C. for 6 to 8 hours; then the layer is dried and stained, and the small colonies are counted under the microscope. Not every cell will have formed a colony at this time, but for practical purposes the results obtained are sufficiently reliable.

Methylene Blue Reduction Test.—A most simple way of grading milk on a bacteriological basis, without making use of intricate bacteriological methods, has become accessible since it was discovered that many stains when added to milk lose their characteristic color sooner or later and are turned white by bacterial action. Among the dyes tested methylene blue has proved most satisfactory. If a cubic centimeter of a highly diluted solution of this dye is mixed with a small sample of milk (10-40 cc.), the latter shows a faint but distinct bluish color that vanishes after a few minutes or after several hours according to the high or low germ content of the milk. The bacteria are said to "reduce" the blue stain to a white leuco-compound, and therefore the test has been called reduction test, although it is in fact hydrogen and other substances pro-

[1] W. D. Frost, *Jour. Infect. Diseases*, vol. 28, 1921, p. 176.

duced by the bacteria or present in the milk itself that cause the change.[1] Pipettes, test tubes, and a water bath kept at 38° to 40° C., is all the apparatus needed for this test.

In the Scandinavian countries, where the city milk supply is very well organized, the methylene blue reduction test has been widely adopted during the last ten years and is now generally recognized as a reliable basis for grading milk according to its germ content, if the grading is done on a broad basis.[2] Individual tests are liable to furnish somewhat

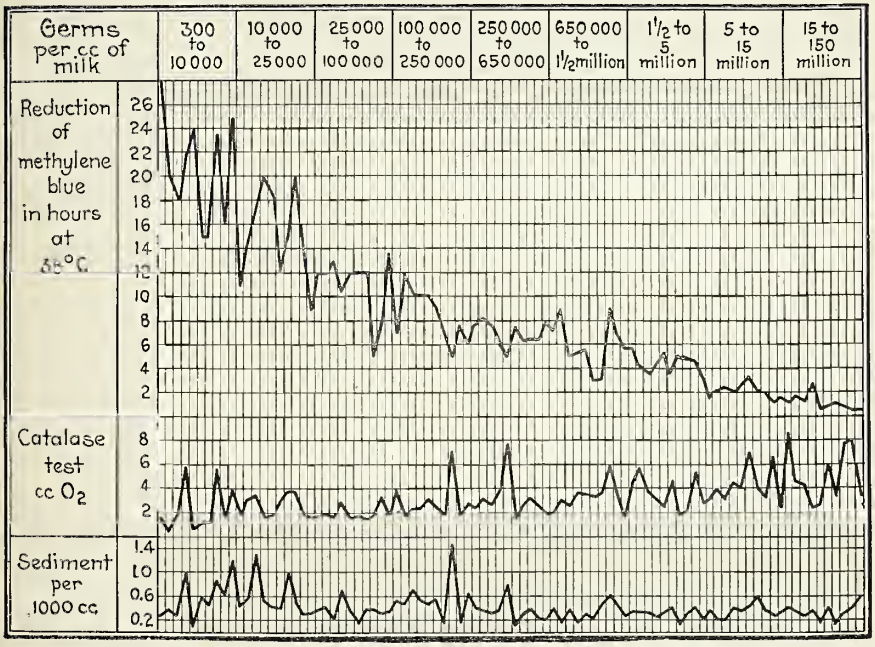


FIG. 43.—Results of reduction, catalase, and leucocyte tests made with 90 samples of milk of different germ content.

conflicting results, but the average of a sufficiently large number of tests, for example the monthly average figure of each milk producer, is generally accepted as very reliable. It is indispensable, of course, that the concentration of the stain added to the milk is always the same. For this purpose tablets are manufactured by the firm Blauenfeldt & Tvede in Copenhagen, Denmark, which are used internationally.

The uppermost curve in Fig. 43 shows what results may be obtained

[1] R. BURRI und J. KÜRSTEINER, *Milchw. Zentralbl.*, vol. 41, 1912, pp. 41, 68; E. B. FRED, *Centralbl. f. Bakt.*, II. Abt., vol. 35, 1912, p. 391.

[2] CHR. BARTHEL und ORLA JENSEN, *Milchw. Zentralbl.*, vol. 41, 1912, p. 417.

in such reduction tests. The general tendency to decline with rising bacterial counts is as conspicuous as is the rather erratic behavior of individual cases, especially if the total germ content is low. There are two reasons for these irregularities. First, the accuracy of the bacteriological counting methods is more or less limited, and in the second place, the different kinds of bacteria act, of course, not uniformly upon the methylene blue or on any other stain. Certain species cause a rapid change, others are slower, and some remain entirely inactive; the influence of variation, age of growth, etc. are of similar importance. This side of the problem has also been very thoroughly investigated by European bacteriologists, and it was noticed that in general the least desirable organisms are most active, that is, not only the quantity but also the quality of the bacteria present in the milk finds an adequate expression in the length of time needed for the reduction. Accordingly, the following classification has been adopted for the routine grading in Scandinavian city milk supplies:

Time of reduction.....	More than 5½ hrs.	5½-2 hrs.	2 hrs.–20 min.	Less than 20 min.
Approximate germ content...	Less than ½ million	½–4 million	4–20 million	More than 20 million
Milk grades.....	I. Good	II. Medium	III. Poor	IV. Very poor

The curve in Fig. 43 shows that even individual tests fit this grouping fairly well. Perhaps the classification might be simplified by drawing the lines at ½, 2, and 6 hours; and the best grade of milk might be singled out by repeating the observation at the end of 12 hours. Undoubtedly the reduction test will gain in importance, the more the bacteriological quality of milk is appreciated.[1]

Catalase Test.—Another biological method for testing milk is the so-called catalase test, which is based upon the fact that peroxide of hydrogen is split into water and oxygen by an enzyme called catalase, present in leucocytes and other cellular elements of the milk as well as in many bacteria. The liberated oxygen is measured, and it is assumed that clean milk is characterized by weak, unclean by strong catalase action. The curve of the catalase tests in Fig. 43 shows that there is indeed a slight rising tendency, indicating the influence of the higher bacterial numbers, but a comparison of the catalase curve with the curve

[1] See also E. G. Hastings, *Jour. Dairy Science*, vol. 2, 1919, p. 293; A. Cunningham and B. A. Thorpe, *Jour. Hyg.*, vol. 19, 1920, p. 107.

for the sediment of the leucocyte test, also given in Fig. 43, makes it evident that the effect of the cell content of the milk is much more pronounced than that of the bacteria. The leucocyte test, however, is much more easily made than the catalase test, and when combined with microscopic tests it is much more valuable, as was explained before.

Acidity and Alcohol Tests.—Because the majority of the bacteria present in normal milk are, as a rule, acid producers, the determination of the acidity of milk by titration, or by measuring the hydrogen-ion concentration, permits a more or less accurate estimate of its total germ content. But alkali and rennet producing bacteria are also not rare, and sometimes they are so numerous that even the spontaneous curdling of the milk will take place while the reaction is still close to neutral, and the hydrogen number not far from normal.

Superior to the acidity test is the *alcohol test.* It consists in mixing in a test tube equal parts of milk and alcohol of 70 or 75 per cent strength, and in ascertaining whether flocculation of the casein takes place. If this happens, the milk will probably coagulate when it is heated. Therefore, this test is of special value when milk is to be pasteurized, condensed, or evaporated.[1] Several or many millions of bacteria are always present in such milk. In addition to the acidity and to the amount of rennet produced by them, the coagulation is dependent on the calcium content of the milk.[2]

Fermentation Test.—Another very practicable and valuable test, which however again requires at least 12 hours' time, has long been used in Switzerland for grading milk in cheese factories. From every delivery samples are filled into four sterilized (boiled) test tubes or jars; two of them receive a few drops of rennet solution, while the other pair remains without rennet. The first-named test (with rennet) is known in America as the Wisconsin curd test. The samples are kept in water of 38° to 40° C. After 12, or better after 24, hours alterations of the milk and characteristic curd formations become visible, such as are shown in Plate X. Furthermore, taste and flavor of the milk are to be tested, and if methylene blue was previously added to the milk (without rennet) the results of the reduction test are secured simultaneously. This combined examination gives to the dairyman a very good general information upon number and quality of microorganisms present, without compelling him to wait for the outcome of detailed bacteriological investigations. If a yellow sediment becomes visible in the fermentation test tube, it may serve as an indication of

[1] U. S. Dept. of Agr., *Bull.* 944, 1921.

[2] H. H. Sommers and E. B. Hart, *Jour. Biol. Chem.*, vol. 40, 1919, p. 1

mastitis milk, analogous to the precipitate in the special mastitis test discussed above.

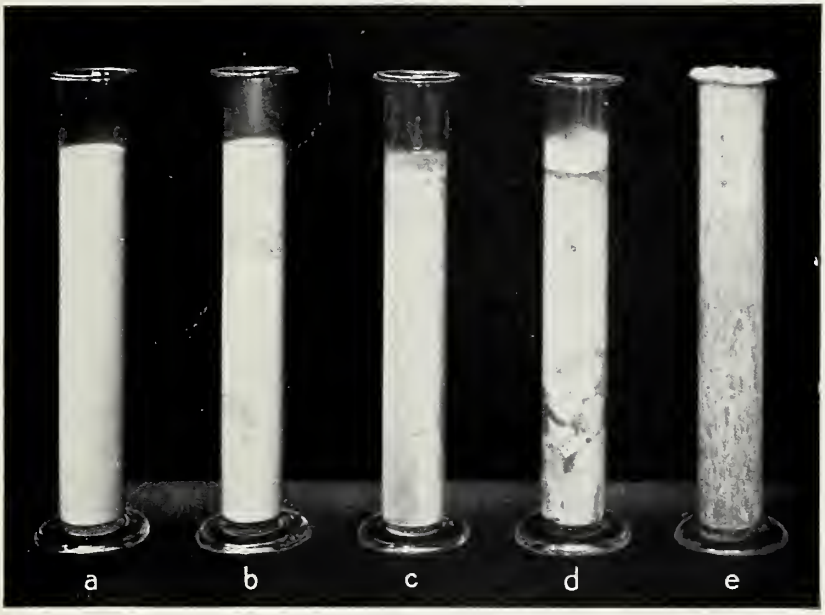
Very clean milk, which remains unchanged in the milk test for 24 hours, gives frequently poor results in the curd test on account of the absence of lactic acid bacteria. If these are present in considerable numbers a homogeneous curd is obtained without, as well as with, rennet. Strong gas formation is nearly always due to fecal contaminations. Fermentation tests made with samples of milk which are taken in sterilized tubes at all places where contaminations possibly occur, will be very helpful in discovering any hidden source of milk infection in the stable or in the dairy.

2. ACTIVITIES OF BACTERIA IN MILK

Not all alterations which may take place in milk are the work of microorganisms. Abnormalities in composition, taste, and flavor are sometimes already noticeable at the moment when the milk leaves the udder, due either to purely chemical influences, as in colostrum and in milk produced close to the end of lactation, or to bacterial infection of the udder, as in mastitis and tuberculosis. If the freshly drawn milk appears normal and the milking is done strictly aseptically, it will often take weeks before composition, taste and flavor are markedly changed. Part of these alterations are caused by genuine milk enzymes, but compared with the enzymatic actions of the bacteria, present even in the cleanest milk, they are of no practical importance. Nearly all the alterations which may develop within the comparatively short time until the milk is consumed, are the work of microorganisms. An exception to this rule is, for example, the unfavorable influence exerted by direct sunlight upon milk fat, which assumes an unpleasant flavor if bottled milk is exposed for some time to bright sunlight.

Normal and Abnormal Alterations of Milk.—The only alteration of milk which may be accepted as normal, and which is even desired in some cases, consists in the formation of lactic acid and the coagulation of the casein. If ordinary milk is plated on whey agar to which chalk has been added, usually a picture like that shown in Fig. 44 is obtained; most colonies produce acid enough to dissolve the chalk in their immediate neighborhood, and a clear halo appears around them.

If sour milk is kept for a few days exposed to the air, at first *Oidium lactis* and other fungi begin to grow on the surface. They destroy part of the acid, and begin to digest the curd. Later proteolytic bacteria may become active, and if the milk is kept long enough, ultimately a



1. Milk Fermentation Tests

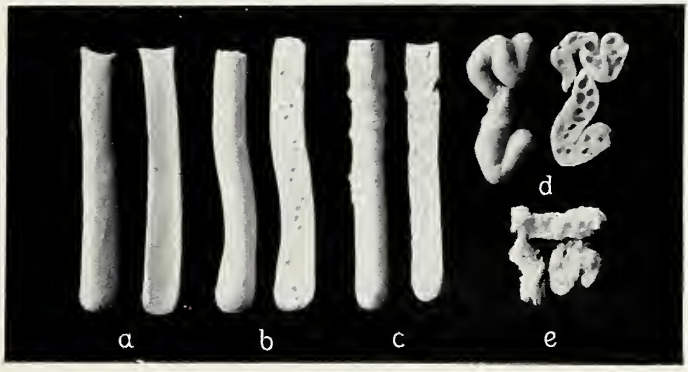
(*a*) Milk remained liquid

(*b*) Gelatinous coagulation, little whey

(*c*) Cheesy coagulation, much whey

(*d*) Strong gas formation (B. coli)

(*e*) Very strong gas formation (B. amylobacter)



2. Curd Tests

(*a*) Smooth, without holes

(*b*) Almost smooth, but with holes

(*c*) Straight but with many holes

(*d*) Blown

(*e*) Torn

(*Facing page 176*)

brownish liquid of offensive odor will result. The absence of air, however, prevents this development; the acid will not be destroyed and no further change will take place.

A rather common abnormal alteration of milk is its curdling under the influence of rennet producing bacteria. Changes in taste, flavor, color, and viscosity occur also from time to time, although they are, as a rule, less frequent now than they were formerly, when little was known about bacterial action, and milk was kept in musty cellars.

Formation of Lactic Acid.—Normal milk always shows a slight initial acidity, due to its chemical composition. The streptococci and micrococci that are almost constantly present in the udders, do not

FIG. 44.—Agar plate with colonies of acid producing bacteria ($\frac{2}{5}$ nat. size).

exert any appreciable influence in this direction, except in very rare cases. The numerous lactic acid bacteria, usually introduced by unsterilized vessels, also remain without effect for some hours. Their multiplication begins at once, because they are not hindered by the weak bactericidal properties of the milk; but not before enough enzymes are produced, will the conversion of the milk sugar into lactic acid become noticeable. Some authors have tried to calculate an "average" efficiency of a single cell of lactic acid bacteria, but it suffices to compare consecutive bacterial counts and acid determinations, simultaneously made during one or several days, to become convinced that no constant relation exists between number and efficiency. For example, the following increases (+) and decreases (—) have been recorded:[1]

[1] O. RAHN, *Centralbl. f. Bakt.*, II. Abt., vol. 32, 1912, p. 375.

Changes after	9	12	15	18	21	42	27 hrs.
Millions bacteria per cc.	+20	+90	+300	+700	+200	+100	−150
Acidity (cc. N/10 NaOH per 1000 cc.)	+1	+1	+4	+22	+20	+5	+2

In other cases the disparity between changes in acidity and in bacterial numbers was even more marked, similar to that shown in Fig. 37 (p. 154). The enzymes survive the dying cells and continue to act; on the other hand, the cells of lactic acid bacteria may lose more or less completely their ability of enzyme production, while they retain their full vitality. In the early stages, about $^4/_5$ of the lactic acid formed enters chemical or physical combinations, especially with milk casein. The hydrogen-ion concentration increases until approximately pH=4.7 is reached, and then it remains at this point until the coagulation of the casein is completed.[1]

Behavior of the Four Groups of Lactic Acid Bacteria.—The main characteristics of the four groups of lactic acid bacteria have been discussed on p. 119, where it was also mentioned that other bacteria may display similar abilities, which however are of no practical importance. It is to be added that some yeasts are known to produce lactic acid, and that occasionally they are active in ripening cream.

According to their *efficiency in lactic acid production* the members of the four groups may be classed as follows. Generally, the smallest quantities of acid are produced by the micrococci; next in activity come the intestinal lactic acid bacteria; stronger and much purer is the lactic acid formation by the streptococci; and the largest quantities are usually produced by the lactobacilli, although a comparatively long time is required before the maximum is reached. 0.5 to 0.8 per cent lactic acid is usually the maximum for the streptococci, 1 to 2 and sometimes 3 per cent for lactobacilli. Naturally, this general classification does not fit every individual case; exceptionally strong and weak varieties may be found in every group.

Whether one or the other group predominates in milk or in dairy products depends on the mode of infection, the temperature, the presence or absence of air, and the composition of the substrate.

Concerning the *mode of infection* it is to be pointed out that the micrococci come mostly from the udder, from the air, and from the utensils; the intestinal lactic acid bacteria, from feces, from impure water, and from unclean vessels; the streptococci, mostly from containers,

[1] L. L. Van Slyke and J. C. Baker, *Jour. Biol. Chem.*, vol. 35, 1918, p. 147.

more rarely from the cow (udder, hair, feces); the lactobacilli, from silage and other food, saliva, feces, soil, and cheese. If the *temperature* is kept below 10° C., micrococci will predominate after a few days; around 20° C. streptococci are very active; 30° to 40° C. are most suitable for the majority of intestinal lactic acid bacteria; and at 45° to 50° C. hardly anything but lactobacilli will survive. This general classification is again not without exceptions, but it is well worth knowing that around 20° and 45° C. the cleanest and most rapid formation of lactic acid is to be expected, while below 10° C. and between 30° to 40° C. many by-products are formed which act unfavorably upon taste and flavor. If milk cultures of lactic acid bacteria are kept continually at high temperatures, practically all of them are killed after a few days, while at 0° C. most cultures will remain alive for a long time, especially if enough chalk is added to neutralize the acid formed. The *presence of air* favors micrococci and intestinal bacteria, while the majority of streptococci and lactobacilli grow best under anaerobic conditions. Their surface colonies on agar plates are therefore small (see Fig. 40), and milk filled into deep containers shows more rapid acidification than that kept in flat vessels. The *quality of milk* is of influence chemically as well as biologically. Variations in the percentage of sugar, casein, and calcium make one milk more or less liable than another to undergo acidification and coagulation. Furthermore, symbiotic and antagonistic effects of the accompanying microflora may stimulate or retard the activities of the lactic acid bacteria. Accordingly, strains of known character can not be expected to act uniformly under all circumstances, even if no spontaneous variation occurs.

Formation of Volatile and Other Acids, of Alcohols, and of Gases.— *Formic, acetic,* and *succinic acids* are almost constantly present in sour milk. The majority of micrococci and of intestinal bacteria are inclined to produce these acids in addition to, or instead of, lactic acid; streptococci and lactobacilli may participate in in the formation of acetic acid, which is occasionally produced in relatively large quantities by the destruction of the lactates first formed.[1] *Butyric acid* appears sometimes in pasteurized and incompletely sterilized milk. Members of the B. amylobacter group are so common in soil, water, fodder, and feces that a few such spores will always gain entrance, but the acid reaction normally produced by the lactic acid bacteria prevents their germination under ordinary circumstances.

The production of *ethyl alcohol* in milk is usually of little impor-

[1] E. B. Fred, W. H. Peterson and A. Davenport, *Jour. Biol. Chem.*, vol. 39, 1919, p. 347.

tance, but in fermented milks, such as kefir and koumiss, 1 to 2 per cent or more may be produced by various yeasts. Sporulating Saccharomycetes as well as non-sporulating Torulaceae are known to become active; some of them attack the milk sugar directly, while in other cases the lactose must first be changed by bacterial action to glucose and galactose, before the alcoholic fermentation can take place. Occasionally some *butyl alcohol* may be produced by butyric acid bacteria.

If *gas formation* becomes noticeable in milk, it is usually an indication of fecal contamination (*B. coli, aerogenes*, more rarely *B. amylobacter*), but during hot summer weather the milk containers are sometimes heavily infested by yeasts, which may cause considerable losses by the foaming of milk and cream in transport, or by fermentations in condensed milk.[1] Gas forming varieties of micrococci, streptococci, and lactobacilli may also become active, although they are not very numerous. It is worth knowing that part of the mastitis streptococci are strong gas producers; therefore, mastitis milk does not infrequently cause gas formation in the fermentation test, as well as in cheese. Intestinal bacteria (B. coli and aerogenes) give a mixture of carbon dioxide and hydrogen, while streptococci, lactobacilli, and yeasts produce carbon dioxide exclusively.

Curdling of Milk.—The coagulation of the casein in milk is not always due solely to the *acids* produced by bacteria; *rennet* of bacterial origin may participate in this process, sometimes it may even act alone. If the milk is rich in true lactic acid bacteria, the curd is solid, smooth, of porcelain-like appearance, and no or little whey of pale color is pressed out (see Fig. 1, b, Plate X). The presence of weak strains is usually characterized by a larger amount of whey. Rennet producing organisms make a soft, loose coagulum, that shrinks gradually; the whey is of yellowish, greenish, or brownish color (see Fig. 1, c, Plate X). Such "cheesy" coagulation of milk is caused by micrococci, different non-sporulating bacteria, such as B. fluorescens, and especially by sporulating bacilli (B. subtilis and mesentericus), whose action is sometimes very troublesome in pasteurized and incompletely sterilized milks. Most frequently acid and rennet combine their effects; many species are known to act simultaneously in both ways. The majority of micrococci are acid and rennet producers; streptococci, non-sporulating and sporulating bacteria may display the same abilities. Because such micrococci are usually present in the udder, acid and rennet coagulation of the milk is so frequent.

[1] O. Hunter, *Jour. Bact.*, vol. 3, 1918, p. 293; B. W. Hammer, Iowa Agr. Exp. Stat. *Research Bull.* 54, 1919.

Sometimes an *abnormally early curdling* of the milk is observed, which is the result of an excessive development of acid and rennet producing cocci within the udder. This may be restricted to individual cases, or all the milk produced may undergo this abnormal change, especially on very hot days with thunderstorms. In the latter case, the effect caused by the contaminations within the udder is usually aggravated by additional contaminations due to less careful milking and handling of the milk, as well as to increased growth of the microflora in washed but not sterilized utensils. However, even if great care is taken, and the milk is thoroughly cooled in order to offset the influence of the hot weather, the increased initial contamination may still cause spoilage. In the production of certified or Grade A milk close observations of these fluctuations of the udder flora are very important.[1]

Decomposition of Casein.—The acid formed in normal milk prevents, as a rule, further decomposition of the casein, but whenever incompletely sterilized milk, or milk with a very low initial germ content, is kept for several days or weeks, a partial dissolution and disintegration of the casein will occur, accompanied by abnormal alterations of taste and flavor; occasionally, even distinctly poisonous substances are produced. The last-named possibility was the reason why the manufacture of so-called sterilized milk could not succeed, although it was considered to be the safest food for children. Spores survive in such milk, and if it is kept for some time, the newly grown bacilli, working under anaerobic conditions, act very unfavorably upon the casein. The partial digestion which becomes visible under such circumstances is not always true peptonization, since peptic enzymes work only if the reaction is acid. A distinctly alkaline reaction is rather frequent in these cases, and tryptic enzymes are produced by the bacteria active in heated milk.

A small amount of *ammonia* is always produced in the course of casein decomposition. Several authors have tried to develop a special test upon this basis, and it is indeed quite probable that low-grade milk will contain more ammonia than can be found in good milk. But no satisfactory method is known at present which would permit rapid and accurate determinations of this kind.

Alterations of the Milk Fat.—Sometimes a partial decomposition of milk fat takes place before the milk leaves the udder or very soon afterwards; such milk is characterized by a more or less rancid taste and flavor. The fat splitting variety of B. abortus, which is not infrequent in healthy udders, may be responsible; several other species,

[1] F. Löhnis, *Molkerei-Zeitg. Hildesheim*, vol. 28, 1914, p. 785.

such as B. fluorescens and related non-sporulating rods, as well as certain micrococci, are capable of acting in a similar manner. Usually, however, no pronounced change of the fat will occur during the comparatively short time the milk is kept, perhaps with the only exception that the cream will be partially oxidized, if bottled milk is exposed to direct sun-light. A "tallowy" taste and flavor is produced in the layer of cream.

Abnormal Taste and Flavor of Milk.—Different kinds of abnormal tastes and flavors occur in freshly drawn milk, mostly at the end of lactation and under the influence of unsuitable food, that are not due to bacterial action; but if the abnormalities develop gradually afterwards, microorganisms are always responsible for such alterations. Sometimes the changes can be correlated with distinct chemical transformations, as in the decomposition of casein and fat, but in other cases it is rather difficult to give an exact definition of the abnormal flavors. Many species of bacteria have been described which were found active in such milk, but practically all of them lost their specific characters when they were grown for a while on artificial substrates. Most of them are to be classed as varieties of B. coli, fluorescens, and proteus, usually originating in the intestines and carried into the milk as fecal contaminations. The special environmental conditions that prevail in the digestive tract under the influence of different feeding, stimulate the appearance of such modifications in the fecal flora. The more fecal contaminations are allowed to get into the milk, the more will the effect of good and bad feed become noticeable in the milk obtained. Accordingly, abnormal taste and flavor of clean milk is due more generally to initial chemical alterations than to secondary bacterial actions.

Pigmentation of Milk.—Pigment producing bacteria have been found occasionally within the udder, but under these circumstances they are unable to produce any color in the milk. A reddish hue, sometimes visible in freshly drawn milk, indicates invariably the presence of blood and a diseased condition of the udder. In all other cases several days will elapse before such pigments will be produced by bacteria, as are shown in Plate VI. It was pointed out on p. 89 that at the present time these organisms and their activities have lost much of their former importance, but a yellow color on cream and a greenish discoloration of milk are not very rare, if milk is kept for some time in cold storage; micrococci or short rods are active in the first case, B. fluorescens in the latter.

If milk is kept in rusty containers, the acids produced dissolve part of the rust. A grayish-blue color and a disagreeable taste are the results of this improper handling.

Slimy and Ropy Milk.—Increased viscosity that makes milk slimy, or in extreme cases distinctly ropy, may be due to the growth of varieties of lactic acid bacteria which have lost part of their ability to produce acid and have increased their inclination to construct slime capsules around their cells. Because slime producing varieties are known to occur in all four groups of lactic acid bacteria, they are nearly as omnipresent as the typical lactic acid bacteria, but since their resistance against unfavorable influences is higher on account of their thick capsules, they may often survive in utensils, wherein the acid producers have been suppressed by cleaning that is not followed by sterilization. The latter procedure kills all slime producers, and to heat all utensils thoroughly by steam, or better in a hot air oven, is the only means by which the trouble can be promptly eliminated. In addition, the source of infection should be investigated; in most cases it is the water or the litter. Adding small amounts of the materials to the milk kept at a suitable temperature, leaves, as a rule, no doubt about the source of infection. As long as the ropiness is not too pronounced, the milk can be used in the household; it is disagreeable, but not unhealthful. Sporulating slime producers and slimy varieties of Oidium lactis may also occur in milk, but they are by no means as common as those varieties of lactic acid bacteria.

Elimination of Abnormal Alterations of Milk.—Whenever abnormal alterations of milk are to be investigated, it must always be kept in mind that the chemical composition of that particular milk may be the main cause why certain bacteria grow so well in it. It is no rare occurrence that laboratory experiments remain inconclusive, because the isolated strains, when tested in pure culture in other milk, which was perhaps sterilized before, fail to display the characters which they have shown before. Local inspections and simple fermentation tests give usually much more satisfactory and more useful results, than are furnished by the isolation of "new species" in the laboratory. These so-called species are in most cases nothing but local varieties of well known bacteria, which have modified their character temporarily, in accordance with environmental conditions. Spontaneous appearance and disappearance of such abnormalities find their explanation in these facts. Utmost cleanliness, thorough sterilization of all utensils, chlorination of the water, and if necessary pasteurization of the milk are to be recommended as practical remedies.

3. MILK PASTEURIZATION—FERMENTED MILKS

Certain effects of bacterial activities in milk could be prevented and remedied by chemical means. For example, increased acidity could be neutralized by the addition of sodium bicarbonate; or peroxide of hydrogen might be added to the milk in order to postpone its acidification. In exceptional cases the use of these two relatively harmless chemicals may be permissible, but as a rule it is far preferable to rely upon careful pasteurization and refrigeration. Pure food laws properly forbid the addition of chemicals to milk.

Milk Pasteurization.—It was pointed out before that the pasteurization of milk by the application of heat reduces the germ content and thus increases the keeping quality of milk. The relatively best method consists in exposing the bottled milk to 63° C. for 20 to 30 minutes, then reducing its temperature by cold water or cold air to about 5° C., and afterwards keeping it below 10° C. Increasing the temperature to 75° or 80° C., as in the so-called flash process, biorization, etc., has no advantage over the pasteurization at 63° C. The chemical qualities of the milk are much less affected by the latter method, but the killing of pathogenic and of other harmful bacteria is just as well realized, provided that the temperature of 63° C. is carefully maintained all the time.

Various *tests* are available which permit to determine whether the heating was sufficient for thorough pasteurization. Because formerly the application of 75° to 80° C. was generally considered necessary for this purpose, most methods that have been worked out thus far, can be used only for the examination of milk pasteurized at such high temperatures. Raw milk contains enzymatic substances whose presence can be proved by certain color reactions, but after the milk has been exposed to 75° to 80° C., these enzymes are more or less inactivated and the color tests give negative results. *Para-phenylen-diamin* and *guaiacol* are the substances most frequently used for such examinations. However, no biochemical test is known that would be applicable to milk pasteurized at 63° C. Only by making *microscopical examinations* is it possible to ascertain promptly whether the heating was sufficient or not. In raw milk the bacteria are easily stained, while the leucocytes, and especially their nuclei, mostly refuse to take the stain, and appear therefore under the microscope white against a darker background. In pasteurized milk the situation is reversed; many of the bacteria are pale, while the leucocytes and their nuclei are darkly stained, their size is generally reduced, and many of the nuclei have been broken up in several parts.[1]

[1] W. D. Frost and G. D. Moore, *Jour. Dairy Science*, vol. 2, 1919, p. 189; W. D. Frost, Univ. of Wis. *Studies* No. 2, 1921, pp. 151–163 with plates.

Proper pasteurization kills approximately 99 per cent of all bacteria growing in milk, so that only a few hundreds or a few thousands will survive. If such milk is promptly cooled and kept below 10° C., its germ content should still be low at the time of delivery. In fact, however, much of the pasteurized milk is very rich in bacteria when it gets into the hands of the consumer, and, as was pointed out before, this secondary growth of organisms is quite different from and much more objectionable than the original microflora. The application of the methylene blue *reduction test* makes the bacteriological examination of pasteurized milk a relatively easy matter, and it seems very desirable

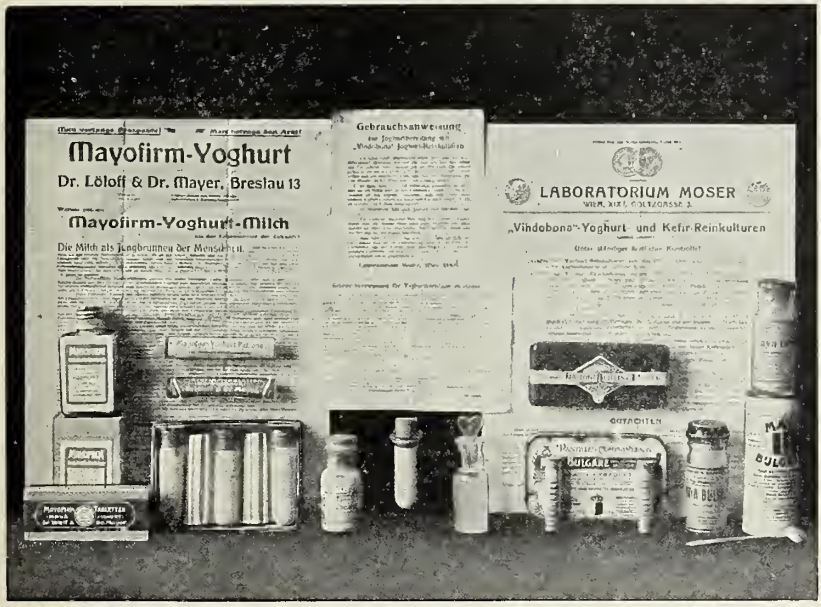


Fig. 45.—Yaourt cultures in powder and liquid form ($\frac{1}{6}$ nat. size).

that extensive use be made of this simple method, in order to eliminate all so-called pasteurized milk whose short reduction time would indicate that it has been improperly handled.

Fermented Milks.—Only a small number of lactic acid bacteria survive pasteurization; therefore it has repeatedly been recommended to add pure cultures of lactic acid streptococci to such milk. A fairly normal microflora would thus be restored and good sour milk could be prepared, if desired. In Eastern European and in Asiatic countries, where lack of cleanliness and high summer temperatures invite early spoilage of all milk, similar procedures have long been in use. The milk

is boiled, sometimes for several hours, and then inoculated with a small amount of fermented milk, or with special preparations which have been found useful for this purpose. Because the milk has to stand rather high air temperatures, the original material used for inoculation is taken quite properly from the stomachs of milk-fed lambs and calves, where lactobacilli adapted to relatively high temperatures grow almost in pure culture. As usual, some symbiotic yeasts accompany the lactobacilli, and they contribute by their alcohol production to the particular flavor of these types of fermented milks. One of them, the so-called *yaourt* or *yoghurt* of Bulgaria, Roumania, Greece, and Turkey is now widely used in Europe and in America. Some of the cultures offered by the trade are shown in Fig. 45; frequently they are of inferior quality. Sour milk prepared along the same lines is called *dadhi* in British India, *matzoon* in Armenia, *lebben* in Syria, Egypt, and

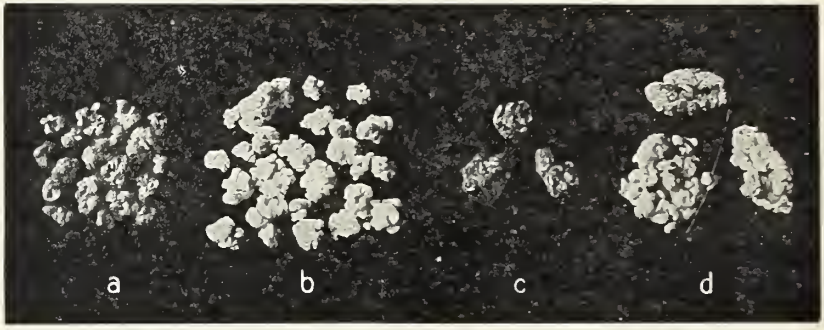


FIG. 46.—Kefir granules ($\frac{1}{2}$ nat. size). Two 1-g. samples dry (*a* and *c*) and fresh (*b* and *d*).

Algiers, *gioddu* or *mezzoradu* in Sicily, *cieddu* in Sardinia, *grusavina* in Montenegro, *huslanka* in the Carpaths. Lactobacilli, lactic acid streptococci, and yeasts are always present and active; local varieties are responsible for the slight, but characteristic differences in taste and flavor.

If large quantities of fermented milk are consumed regularly, as is done by those primitive people, the lactic acid bacilli will suppress most other bacteria in the intestines; putrefaction and toxin production will cease almost completely, and frequently an improvement in general health will result. But it is not so much the bacteria introduced with the milk, as the milk itself that acts favorably. Babies, calves, and lambs are not fed fermented milks, and yet their digestive tracts contain hardly anything else than lactobacilli. Milk inoculated with calves' feces and kept at 45° C., gives after a few transfers a "yaourt," as

good as, or better than any one imported from the East. Accordingly, it has also been tried to improve the intestinal flora in man by feeding pure cultures of *B. acidophilus,* that is, a lactobacillus variety most common in the digestive tract of milk-fed babies. But again it was observed that the desired change was secured only if regularly large quantities of milk or of milk sugar were consumed, and in this case B. acidophilus establishes itself spontaneously, as it does in every child.

There are a few other fermented milks which have also been recommended for therapeutic purposes. They are the *koumiss* and the *kefir,* both of Russian origin and both characterized by their relatively high content of alcohol (1 to 2 per cent) and of carbon dioxide. Again streptococci, lactobacilli, and yeasts are working together, but the latter are much more active in these cases. Genuine koumiss is always made of mares' milk which is relatively rich in sugar, and therefore especially liable to undergo an alcoholic fermentation. By distilling koumiss highly intoxicating liquors are manufactured by Siberian tribes. The microorganisms of kefir form quaint berry-like agglomerations, which have been widely sold in Europe and in America when the use of kefir was in vogue. In Fig. 46 they are shown in the dry, shrunken condition as they come in the trade, and in the swollen, soaked state which they assume in milk.

A third type of fermented milk is prepared in the Scandinavian countries, in Holland, and in Northern France, where the air is generally cool, and the streptococci are therefore more inclined to overgrow the lactobacilli and yeasts, although these too are present. The variety of streptococci predominating in these milks produce lactic acid, as well as fairly large quantities of slime, and these milks show therefore, after they have curdled, a peculiar "tight," gelatinous consistency, which is the reason why they are called in Norway and Sweden *taette* and *taettemjölk,* that is, tight milk. The *fiili* or *püma* prepared by Finnish settlers in Minnesota is another example of this type of sour milk.

CHAPTER X

BACTERIA AND RELATED MICROORGANISMS IN BUTTER

Butter contains on the average 80 or more per cent of milk fat, about 16 per cent of water, and small quantities of casein and of salt, if this is added. The salt used is equivalent to 2½ to 3 per cent of the total weight; it dissolves in the water and changes this to a rather concentrated brine. Pure fat, such as lard or other commercial products, is almost completely resistant against attacks of microorganisms, because they can not live on fat alone. If butter is melted and carefully freed from all non-fat by skimming and decanting, the purified butter fat may be kept practically unchanged for many months. In normal butter, however, the casein supplies the necessary nitrogenous food for numerous microorganisms, and if they are checked by the added salt or by low temperatures, their enzymes will continue to act favorably or unfavorably upon taste and flavor of the butter. Extensive investigations of the last twenty or thirty years have made it clear that the careful control and regulation of the microflora of the butter is of great practical importance.[1]

1. GERM CONTENT OF BUTTER

The germ content of butter may be low or high, because it is influenced by several circumstances that are highly variable. The microflora of the cream is of prominent importance, which in its turn is dependent on the milk, and on the changes occurring during separation, transportation, pasteurization, and ripening of the cream. The methods, materials, and utensils used for butter-making add their specific influences. Careless treatment, impure water, low-grade salt, and unclean utensils will necessarily injure the quality of the product. Further changes in the microflora of the butter take place while it is stored or shipped, and when the butter reaches the consumer many alterations may be noticed which could have been prevented, if proper attention had been paid to the possible effects of bacterial action.

[1] For references see F. Löhnis, "Handbuch der landw. Bakteriologie," Chap. III, 2.

Microorganisms in Cream, Water and Salt.—Because of their relatively large size and great number, the fat globules in milk carry comparatively large quantities of bacteria into the *cream;* accordingly, the germ content of cream is always higher than that of the milk used. Pasteurization kills approximately 99 per cent, but rapid multiplication takes place in the ripening process. Hundreds of millions of bacteria are regularly present in ripened cream, and if this is allowed to become very acid, one or several thousand millions will be found, mostly lactic acid bacteria, but always accompanied by a variable number of other species.

The *water* used for rinsing the utensils and for washing the butter, should be boiled or otherwise sterilized, unless its germ content is unusually low. B. fluorescens, slime producing bacteria, such as Bact. lactis viscosum, and spores of B. amylobacter are common in water, and if they get into the butter in considerable quantities, a distinctly unfavorable effect will result.

Salt, too, is sometimes very rich in microorganisms which are able to withstand the antiseptic effect of the sodium chloride, and may cause a marked deterioration of the quality of the butter. Only salt should be used that is absolutely pure, bacteriologically as well as chemically; chemical impurities, such as traces of iron compounds, may act as unfavorably upon the taste of the butter, as may be the case with microorganisms.

Influence of Utensils, Air, and Paper.—Aside from the microorganisms brought into the butter with the materials used in its manufacture, other forms are added by the contact with bacteriologically unclean *utensils.* Metal is not very suitable for churns, etc., because the delicate flavor of butter is easily impaired in such containers; wooden churns and utensils are generally preferred, despite the fact that they are more difficult to clean and to sterilize. For such churns the application of dry heat is impossible, and steaming proves also detrimental to the structure of the wood. Some kind of chemical treatment must be relied upon; milk of lime has proved suitable for this purpose.

The *air* in rooms where butter is made and kept, should be free of microorganisms which might grow on the butter and injure its quality. Mold spores come first in this respect; if necessary, they should be killed by fumigation.

The *paper* used for wrapping the butter may act favorably or unfavorably upon its quality. Good paper protects the butter against the invasion of molds, but paper of inferior quality, that is, paper which is comparatively rich in soluble organic substances, such as dextrin and glycerol, favors the growth of microorganisms upon the butter, even if

it does not itself carry additional contaminations. Previous boiling of the paper in a 25 per cent brine removes such substances, and at the same time kills all microorganisms that may adhere to the paper.

Changes in Germ Content During Storage.—If butter is made from pasteurized sweet cream its initial germ content is low, but within the first few days a rapid increase takes place, due to the multiplication of lactic acid bacteria, which is followed by a gradual decline. If ripened cream is used, the lactic acid bacteria have already grown therein, and the maximal germ content is reached at the beginning; only in cases where the microflora of the sour cream was very mixed, a slight increase of the total number may occur during the first few days. The gradual dying of the microorganisms in old butter is always noticeable. The following figures represent typical counts:

Millions per g. Butter		Freshly Made	After 1 Week	After 2 Months
Butter made from	pasteurized sweet cream..	1	10–30	0.6–2
	ripened cream.....	10	3– 8	0.1–2
	impure sour cream.....	20	5–40	0.4–2

The lower figures recorded after 1 week and 2 months were obtained with material taken from inner parts, whereas the higher figures always refer to the bacterial growth on the surface of the butter. The microorganisms active in the decomposition of the fat are all aerobic; the casein which they also attack furnishes ammonia and other products of alkaline reaction, and the resulting partial neutralization of the acidity favors the continued growth of the lactic acid bacteria, which are rapidly killed by the acid in the inner part of the butter.

If the butter has not been thoroughly freed from casein, its germ content may reach 100 millions per gram and more, while the best grade of butter made for the export trade is characterized by exceptionally low bacterial counts. The absence of air in the containers prevents any rise in numbers. For example, the following counts were obtained from such material:

Microorganisms in 1 g. butter	After 1	2	3	18 weeks
	362,000	125,000	23,600	200

Types of Butter Organisms.—Almost without exception *lactic acid bacteria* make up the vast majority of butter organisms. Among them nearly always the streptococci predominate, especially if the cream was ripened at a temperature in the neighborhood of 20° C. If

it was kept at lower degrees, the lactic acid micrococci become more conspicuous; the same holds true in regard to the influence of cold storage. Lactobacilli are also present; they grow slowly in the butter, but after a few weeks they may survive the short-lived streptococci. Low-grade butter is comparatively rich in *B. coli* and *aerogenes.*

Other non-sporulating and sporulating bacteria, such as *B. fluorescens, B. amylobacter, subtilis,* and *mesentericus,* are found quite regularly, though not in great numbers. Their influence upon the quality of the butter is sometimes very marked.

Yeasts, molds, and *actinomycetes* are rather common in butter of inferior quality. A marked increase in yeasts is frequently noticeable in old butter. Molds were found to be more numerous in margarine than in butter.

Pathogenic microorganisms may be carried by butter, as well as by milk, but thorough pasteurization of the cream and strict observance of all rules concerning the prevention of the spreading of pathogenic organisms, especially with regard to healthy disease carriers, will eliminate this possibility. Grass bacilli are not infrequent in butter, where they are sometimes mistaken for tubercle bacilli.

2. BACTERIAL ACTION AND QUALITY OF BUTTER

Taste and flavor of butter is dependent, in the first place, upon the quality of milk and cream. It should be remembered that the specific influence exerted by different foodstuffs is frequently more pronounced in butter than in milk, because the milk fat is much more easily affected than are the other milk constituents. "Grass butter" made in summer is rather different from the "straw butter" produced in winter; the effects of good and bad silage, of beet tops, and of some of the concentrated foodstuffs are very marked. Nevertheless, good butter can be made from all such milk, provided that the cream is thoroughly pasteurized, well ripened, and the butter is carefully made. Slight changes in the treatment of cream and butter may help to reduce or to eliminate certain unfavorable influences of the milk; the microorganisms growing in cream and butter are used in this case for improving the quality of the butter. However, there are other possibilities where bacteria or fungi may act unfavorably upon the butter, and their enzymes may continue these harmful activities after the organisms themselves have died.

Influence of Microorganisms upon Taste and Flavor.—When pure cultures of lactic acid bacteria were first used for ripening pasteurized cream, the objection was frequently raised that such butter was lacking

in flavor, although its better keeping quality was generally acknowledged. Naturally, the mixed microflora growing in home-made starters, caused a much more varied activity in the butter, resulting in a stronger flavor, but at the same time in a quicker deterioration of the product. Gradually the cleaner though milder taste and flavor of butter made with pure cultures was more and more appreciated. Attempts to add special types of aroma producing microorganisms to the starters have been made repeatedly, but most of these experiments were unsuccessful; for a while the selected strains continued to act favorably, sooner or later, however, they changed and began to show distinctly unfavorable features. Permanently satisfactory results have been secured only with certain strains of lactic acid streptococci, of which it was first shown by the Danish dairy expert Storch that they may exert either none, or a favorable, or an unfavorable effect upon taste and flavor of the butter. Volatile acids produced from the lactates first formed, or from the citrates originally present in milk and cream, are largely responsible for the desired flavor of butter.[1] Skillful selection and propagation of good strains of such organisms have become important tasks of dairy bacteriologists; the great and often unexpected influence of symbiotic action must also be carefully considered in such cases.

Abnormal Flavors.—Some strains of lactic acid streptococci produce peculiar oily tastes in butter, as was pointed out by Storch in Denmark about thirty years ago. More frequently, however, such abnormalities as oily, fishy, and rancid taste and flavor are due to the decomposition of the butter fat. Liberation of trimethyl-amine from lecithin may act as another cause of fishy flavor.[2] A peculiar "cheesy" or "sour" taste is sometimes created by a heavy growth of lactobacilli, which is frequent in cream that was not promptly cooled after pasteurization. As usual, yeasts are simultaneously present in such butter; if their number is large, a "yeasty" flavor will appear. If the casein is strongly attacked by proteolytic bacteria a distinctly bitter taste may become noticeable, or it may be merely more or less "impure," when the decomposition has made less progress. The longer the butter is kept, the more will its flavor deteriorate, due to the combined action of microorganisms and of their enzymes upon all the constituents of the butter.

Thorough pasteurization of the cream and inoculation with pure cultures of lactic acid bacteria will prevent and retard many of these undesirable changes. But since new contaminations with such common

[1] B. W. Hammer, Iowa Agr. Exp. Stat. *Research Bull.* 63, 1920.
[2] G. C. Supplee, Cornell Agr. Exp. Stat. *Memoir* 29, 1919.

organisms as B. fluorescens, Oidium lactis, Penicillium glaucum, and other molds, all of which act unfavorably upon the butter, can not be avoided entirely, special methods must be applied in order to protect the butter which is not consumed within a short time. In addition to the removal of the milk proteins by washing, the addition of salt plays an important rôle in this respect. Butter containing 2½ to 3 per cent salt is almost completely fortified against the fat-splitting B. fluorescens and many other bacteria; the fungi, however, will continue to grow, if they are not checked by the absence of air and by the application of low temperatures, as in cold storage.

Deterioration of Butter in Cold Storage.—At a temperature close to 0° C. psychrophilic bacteria are still active; they die slowly at lower temperatures, but the enzymes produced by bacteria and fungi may continue to act. If the butter to be placed in cold storage is carefully made from thoroughly pasteurized sweet cream, none or very little deterioration is to be expected. Ripened cream always carries enough bacterial enzymes to cause sooner or later marked changes in taste and flavor, and if traces of metal had been dissolved by the acids a disagreeable metallic flavor will appear. Furthermore, such traces of metal may hasten the deterioration of the butter by stimulating the enzymatic action; in this respect copper salts have proved most effective.[1] Spontaneous oxidation of fat and milk sugar contributes to the inferior flavor of cold storage butter, if it is kept at a temperature close to 0° C.; but at very low degrees (—18° C.) it is practically of no influence.[2]

Rancidity of Butter.—If butter is made without pasteurization of the cream and without pure culture starters, its very mixed microflora always contains many fat-splitting organisms that will quickly cause rancidity, especially if the temperature is high. Micrococci, B. fluorescens, prodigiosus, and many molds are active in such butter; among the latter a species called *Cladosporium butyri* is most characteristic of rancid butter. All these organisms are aerobic, and they attack fat as well as casein; accordingly, they are most active at the surface of the butter. The zone occupied by them is marked by its darker, more transparent appearance, due to the disintegration of the casein.

Because of the complex nature of butter fat different volatile and non-volatile fatty acids, as well as glycerol, are liberated by the fat-splitting organisms. The glycerol is quickly transformed by the same or by other bacteria and fungi, and various intermediate products appear. Of these, butyric acid esters are typical constituents of rancid butter.

[1] O. F. Hunziker and D. F. Hosman, *Jour. Dairy Science*, vol. 1. 1917, p. 320.
[2] D. C. Dyer. *Jour. Agr. Research.* vol. 6, 1916. p. 927.

The free acids present in such butter can not be accepted as an accurate measure of its rancidity, although frequently investigations upon this subject have been restricted to such determinations.

Decomposition and Oxidation of Fat.—When butter becomes rancid the bacterial decomposition of the fat is frequently accompanied by a spontaneous oxidation of the fat. For a long time both processes have not been properly separated, and many incorrect ideas have been promulgated. Oxidation takes place even with the purest sample of fat if this is exposed to higher temperature or to direct sunlight in the presence of air. If a piece of fresh butter is exposed to sunlight, it can be easily ascertained that this purely chemical process leads to results quite different from those produced in rancid butter by the fat-splitting microorganisms. If part of the butter is protected by wrapping paper, the alterations of the exposed part will be clearly noticeable.

The color of oxidized butter is lighter, not darker as in rancid butter; the taste is "tallowy," not rancid; the splitting of fat and the transformation of glycerol are strong in rancid, but very weak in oxidized butter; butyric acid esters are present in rancid butter, while in tallowy butter aldehydes are frequent. The white color and the abnormal taste of oxidized butter are sometimes so conspicuous that occasionally such butter has been declared to be adulterated with other fats. Low temperatures and the absence of air prevent spontaneous oxidation, as well as the bacterial decomposition of butter fat.

Abnormal Consistency and Discoloration of Butter.—If butter is unusually soft or otherwise of abnormal consistency, the cause will be found, as a rule, in the application of faulty methods. Occasionally, however, slime producing and proteolytic organisms are so numerous in the cream that difficulties arise when the butter is made, and the product obtained is of soft texture and of poor keeping quality. Proper pasteurization will eliminate this trouble. Sometimes a rapid deterioration of butter has been found to be due to a heavy growth of anaerobic butyric acid bacteria; the gas produced by these organisms makes numerous small holes in the butter, looking like pin pricks. The spores of these organisms can not be killed by pasteurization, but since they do not germinate in an acid substrate, vigorous lactic acid formation serves as a remedy. However, the use of clean milk and clean water will prevent contaminations of such kind.

The color of butter is always dependent on the natural color of the milk fat, which varies according to the food of the cow. Abnormal pigmentation, such as yellow, red, green, brown, and almost black discolorations, may be caused by various contaminating microorganisms, mostly yeasts and molds, which usually can be excluded without great difficulty.

Paper and containers are generally the source of such growth; it was mentioned before how paper of unsatisfactory quality should be treated. Irregular white spots and stripes, sometimes visible in the inner parts of butter, are not caused by bacterial or fungous growth, but by an irregular distribution of salt and water.[1]

3. CREAM PASTEURIZATION—USE OF STARTERS

It is undoubtedly possible to get satisfactory results without pasteurization of the cream and without the use of a high-grade starter. However, the results obtained in this way may also be very unsatisfactory. The best mechanical treatment of the butter is not sufficient to secure regularly a product of uniform, high quality; for this purpose the biological requirements must be fully considered.

Cream Pasteurization.—The application of high temperatures (85° to 90° C.), which can not be recommended for the pasteurization of milk, is very suitable for the pasteurization of cream in the making of butter because of the following reasons. In the first place, the accidental microflora of the cream is usually very mixed, and it is desirable to eliminate it as completely as possible. In the second place, the high temperatures inactivate the bacterial enzymes which otherwise would continue their harmful activities in the butter, if this is placed in cold storage. The relatively high percentage of fat and non-fat solids in the cream reduces to some extent the efficiency of pasteurization; it was observed, for instance, that part of the lactobacilli may survive the temperature of 90° C. in cream, while they are promptly killed in milk, if this is pasteurized at such high degrees. However, comparatively few lactic acid bacteria will survive this treatment, and it is absolutely necessary, in order to restore the proper microflora to cream and butter, that a good home-made starter, or one prepared from a commercial culture, is added to the pasteurized cream. Without this protection cream and butter would be open to the invasion of many detrimental organisms. It has been tried repeatedly to replace the lactic acid bacteria by adding pure lactic acid or a mixture of different acids to the pasteurized cream, but the results obtained were very unsatisfactory.

Use of Starters.—Sour milk or butter milk of clean taste and flavor have long served and are still used as home-made starters, but at present it is generally more satisfactory to prepare pure-culture starters by inoculating pasteurized skim milk or milk-powder solution with a reliable commercial culture. As shown in Fig. 47, these cultures are sold either

[1] O. F. Hunziker and D. F. Hosman, *Jour. Dairy Science*, vol. 3, 1920, pp. 77–106

in liquid form or as a dry powder. Liquid cultures are usually purer and more active, but less durable than those in powder form, therefore, the former are preferable for immediate use, while the latter may be kept for emergencies. The labels of the cultures should always show the date when the cultures have been made, so that the buyer is sure to receive a fresh, vigorous culture; the directions furnished with the cultures should be followed carefully. Most cultures contain, besides contaminations, nothing but lactic acid streptococci; by mixing strains that act favorably upon taste and flavor of the butter very satisfactory results may be se-

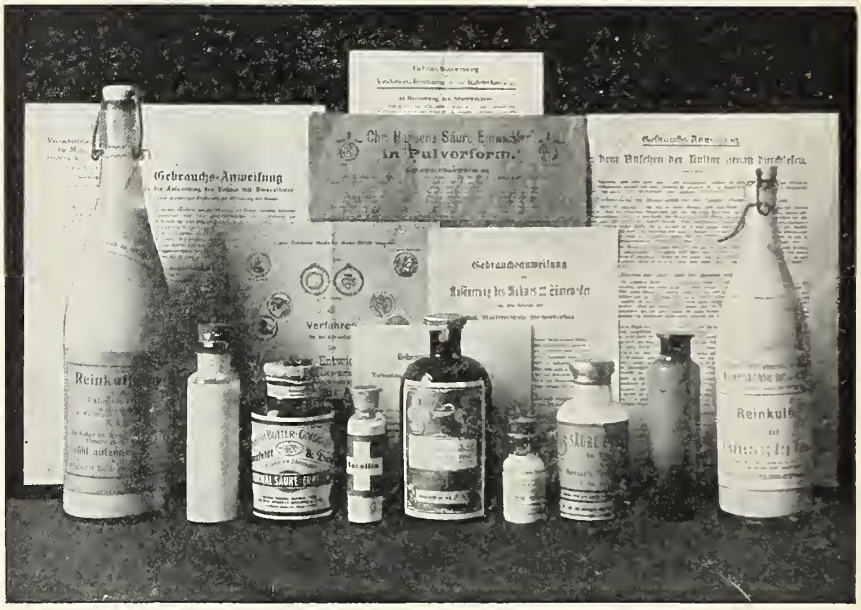


FIG. 47.—Commercial cultures for cream ripening.

cured. In some cases yeasts are a regular constituent of the commercial culture; they, too, are supposed to improve the flavor of cream and butter.

The catalase test, of which it was said (p. 174) that it is not of much value for the bacteriological control of milk, can be used as a simple and reliable means of testing the purity of starters, because the lactic acid streptococci display hardly any catalytic abilities. Strong gas formation in the test indicates the presence of contaminations, which should be checked before they have time to establish themselves in the mother starter and to act unfavorably upon cream and butter.

CHAPTER XI

BACTERIA AND RELATED MICROORGANISMS IN CHEESE

In milk a very low germ content is most desirable; the quality of butter is best if its microflora is composed almost entirely of lactic acid streptococci; but in cheese always very many and very different microorganisms are to be found, because they participate actively in the various biochemical processes which in their entirety constitute the so-called ripening of cheese.

The many kinds of cheeses which are on the market owe their differences to the fact that the special technique applied in each case creates environmental conditions which favor certain associations of microorganisms whose activities produce the characteristic quality, taste and flavor of the cheese. It is easily understood that curd taken from sour milk presents conditions rather different from those prevailing in curd that is prepared by adding rennet to sweet milk. The high water content in soft cheese and the relatively low percentage of moisture in hard cheese influence the bacterial action very markedly. In small flat cheeses, like those of Camembert and Brie, the conditions are more aerobic, while they are distinctly anaerobic in the large heavy loaves of Swiss or Emmental and Cheddar cheese. The special technique in the preparation of milk and rennet, in the treatment of the curd, in forming, pressing, and curing the cheese favors certain and suppresses other groups of microorganisms. Much valuable knowledge upon all these problems has been accumulated during the last thirty years,[1] but many special problems require continued investigations in order to secure a completely satisfactory scientific basis for the much-varied practice in making and curing cheese.

1. GERM CONTENT OF CHEESE

Examination with the naked eye shows that in hard cheeses of Cheddar and Emmental type no growth of microorganisms becomes visible; they contain nothing but bacteria. In soft cheeses, however, various fungi may be seen upon the surface, as on Camembert and Brie,

[1] For detailed references see F. Löhnis, "Handbuch der landwirtschaftlichen Bakteriologie," Chap. III, 3.

or inclosed within the curd, as in Roquefort, Stilton, and Gorgonzola. Their number and kind depend originally on the germ content of the materials and utensils used for preparing the cheese, but many secondary alterations occur during the time in which the cheese is cured; the more time is consumed, the greater is the reduction in living cells.

Origin of Cheese Organisms.—Milk and home-made rennet, if this is used, are the main sources of the microflora of cheese. If the milk is kept until it reaches a certain degree of acidity, before the rennet is added, or if it is allowed to curdle spontaneously, hundreds or thousands of millions of bacteria, mostly lactic acid streptococci, are inclosed in every gram of curd. If cheese is made by adding a home-made rennet infusion to sweet milk, the bacteria in the rennet are of prominent importance. Such infusions contain usually about 100 millions per cc., and approximately 1 million of rennet bacteria is added to each cubic centimeter of milk, while commercial rennet preparations in powder or tablet form carry only a few contaminating organisms. However, the quality of the microflora of the milk plays an important rôle also in those cases where rennet infusions are used, and this holds true especially in regard to the udder bacteria as well as to those added as fecal contaminations.

The influence of the germ content of water, air, and utensils is almost negligible insofar as numbers are concerned, but occasionally organisms from this source may become responsible for certain abnormal alterations occurring in cheese, and the molds growing normally on and in various kinds of soft cheese were also originally derived from these sources, although at present they are frequently added as pure cultures.

Pathogenic organisms may sometimes be carried by soft cheeses which are cured within a few days or weeks, while they will die in hard cheeses during the long ripening period. Milk pasteurization will exclude any danger from soft cheeses, provided that no disease carrier was allowed to touch the products.

Frequency of Microorganisms in Cheese.—When milk coagulates, the majority of its bacteria, approximately 70 to 80 per cent, are inclosed in the curd. At this time the germ content of sweet milk cheese is usually lower than that of sour milk cheese, and if the curd of the sweet milk cheese is heated to a rather high temperature, as is done with Swiss or Emmental cheese, the numbers are still further reduced; but soon a rapid multiplication sets in. After a few days a maximum is reached in the inner parts, which is followed by a persistent decline, similar to that noticeable in butter, while on the surface a luxuriant growth may establish itself, provided that it is not checked by a special treatment of the rind. The following data may serve as an illustration:

Millions per g.	Emmental Cheese		Cheddar Cheese	Sour Milk (Harz) Cheese	
	Surface	Inner Parts	Inner Parts	Surface	Inner Parts
Fresh curd.....	.....	8	10	....	..
After 1 day	.....	450	30	70	70
6 days.....	.....	...	..	300	23
10 days.....	8,500	62	41	....	..
13 days.....	.....	...	..	100	82
45 days.....	66,000	55	10	....	..
150 days.....	23,000	34	0.5	....	..

The white slimy growth appearing upon the surface of young sweet milk cheeses was found to contain up to 500,000 millions of bacteria per g.

If the curing takes place at low temperatures, as with American Cheddar cheese, the reduction in numbers is much slower than at higher temperatures, as is shown by the following counts:

Millions per g.	After 1 Day	After 15 Days	After 70 Days
At 18° to 20° C.....	523	145	4
At 3° to 5° C.....	523	489	473

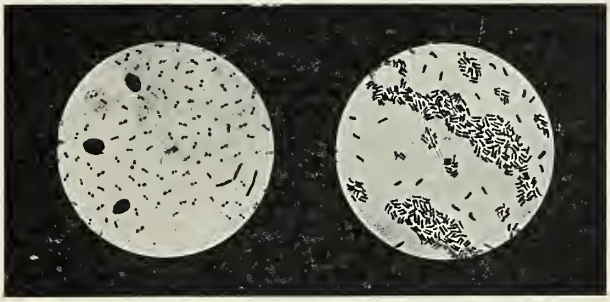


FIG. 48.—Contact preparations (×600) made from Gervais (left) and from Scotch Cheddar cheese (right).

The *distribution* of the bacteria is fairly uniform in young cheeses, but later colonies are formed which act as "ripening centers." It is easily understood that reliable plate counts are hard to obtain from such material, and that direct microscopic tests furnish much more accurate and complete information. Figure 48 shows such stained preparations made from Gervais cheese, a French soft cheese, and from Scotch

Cheddar cheese. In the first case, besides a few yeasts and rods, only lactic acid streptococci are visible in uniform distribution, while in the second case the characteristic accumulation of lactobacilli is very pronounced.

Species of Cheese Organisms.—*Lactic acid bacteria* are always most abundant in sweet milk as well as in sour milk cheeses. Close to the surface many aerobic lactic acid micrococci are to be found, while streptococci and lactobacilli are most numerous in the center, due to their tendency toward anaerobic life. In soft cheeses as well as in young hard cheeses streptococci are much more numerous than lactobacilli, but these predominate always in ripe hard cheeses. The Scotch Cheddar cheese used for Fig. 48 was made with a starter that contained nothing but lactic acid streptococci, yet this type of lactic acid bacteria was practically absent in the ripe cheese. With American Cheddar cheese analogous results have been obtained.[1] The same change was observed in Swiss cheese prepared with rennet powder instead of home-made infusion. The percentage of both groups of lactic acid bacteria was found to be as follows:

Percentage	In the Fresh Curd	After 1 Day	After 17 Days
Streptococci	100	70	0
Lactobacilli	0	30	100

Home-made rennet infusions are always rich in lactobacilli, because these predominate in the digestive tract of milk-fed calves. On the other hand, the intestinal lactic acid bacteria (B. coli and acrogenes) appearing as fecal contaminations in cows' milk may become distinctly harmful in cheese, because of their ability to produce gases and to exert an unfavorable influence upon taste and flavor of the product.

Other non-sporulating and sporulating bacteria are generally of very little importance. In a few cases they may participate in the ripening process, but these are rather rare exceptions.

Yeasts, the habitual symbionts of lactic acid bacteria, are quite common in cheeses. They are absent only if the curd is heated to 55° C., as in making Swiss cheese, because this is their lethal temperature.

Molds are of importance in certain soft cheeses, while they are sup-

[1] E. G. Hastings, A. C. Evans and E. B. Hart, *Centralbl. f. Bakt.*, II. Abt., vol. 36, 1913, p. 457.

pressed in other soft cheeses, as well as in all hard cheeses whose surface is treated in a manner that prevents the development of such fungi.

2. BACTERIAL ACTIVITIES AND THE RIPENING OF CHEESE

If cheese is prepared aseptically or is made from sterilized curd, it will show but restricted alterations which are very different from those characteristic of normal ripening. Microorganisms and enzymes are indispensable for obtaining the desired transformations, which extend to all —nitrogenous as well as non-nitrogenous—constituents of the cheese. In the literature, the term ripening was repeatedly used as synonymous with metabolism of nitrogen only, and many misunderstandings have arisen from the ambiguous use of that term. Undoubtedly, the transformation of casein and of other nitrogenous compounds represents the most important part of the whole complex of chemical changes, but if the curd is freed from milk sugar by thorough washing, the decomposition of casein gives rise to typically putrid odors, although the chemical analysis will furnish results not very different from those obtainable with normal cheese. Therefore, a correct insight into the chemical and bacteriological problems of cheese ripening is assured only if comprehensive investigations are made of all chemical changes occurring in the ripening cheese; besides the metabolism of casein, the transformation of milk sugar and of fat are of foremost importance.

Acid Formation.—In hard cheeses, from which most of the whey is removed by pressing, all milk sugar is usually transformed into acids within 8 to 10 days, while in soft cheeses the higher whey and sugar content leads to a prolongation of this process. The large loaves of hard cheese are soon free from all oxygen; accordingly, the lactic acid streptococci and lactobacilli find most favorable conditions. The intensity of acid formation in the whey, immediately before and after the cheese is formed, determines to a large extent the regular course of ripening. Every type of cheese has its characteristic acidity curve, which has been most carefully studied in Cheddar, Swiss, and some of the French soft cheeses. The total titratable acidity is equivalent to 1 to 1½ per cent of lactic acid in hard, and to 2 to 4 per cent in soft cheeses, but because the acidity of casein and of acid phosphates influences the results so obtained, these figures are only of relative value, and do not represent the real amount of lactic acid present in the cheese. Especially in old hard cheeses frequently all free acid is gone, as is shown by determinations of their hydrogen-ion concentration.

If the acid formation is too weak, the casein decomposition becomes

too intensive, and gas producing bacteria may become very active, while an abnormally strong acid formation will suppress the desired nitrogen metabolism, and no typical ripening will take place.

Transformation of Lactic Acid.—The free acid first formed is more or less completely neutralized by casein, calcium, and the ammonia liberated in the decomposition of the organic nitrogen compounds, and the lactates are further changed to volatile acids and carbon dioxide. The reduction in acidity has been ascertained by numerous titrations, but a more accurate insight will be gained if these experiments are repeated and the hydrogen-ion concentrations determined. For example, the titratable acidity, calculated as lactic acid, showed the following behavior in Limburger cheese:

	Ripe Cheese	
Young Cheese	Inner Parts	Surface
3.21 per cent	1.35 per cent	0.89 per cent

Probably the hydrogen number would have shown that no acidity remained in the surface part of the cheese. The numerous microorganisms living in this layer produce much ammonia for neutralization, and they oxidize the lactates, so that an alkaline reaction soon results.

Formic, acetic, and propionic acids are very common products of the transformation of lactates. Swiss cheese is exceptionally rich in propionic acid; the bacteria active in this case are of great influence upon the texture and flavor of first-class Emmental cheese. Part of the volatile acids forms esters with the small quantities of ethyl alcohol, produced simultaneously by different bacteria and by yeasts, if these are present; such esters are also of considerable influence upon taste and flavor of the cheese.

Decomposition of Casein.—That the decomposition of casein proceeds rather differently in hard and in soft cheeses is evidenced by a superficial examination of the various kinds, after they have well ripened. The texture of the curd shows that a greater percentage of casein is changed to soluble compounds in the soft cheeses, which when overripe may even assume a semi-liquid consistency. Their stronger flavor indicates at the same time that more ammonia is produced than in hard cheeses. In the latter only $^1/_3$ to $^1/_2$ of the total nitrogen is found in the form of soluble compounds, and these are present chiefly in the form of certain amino acids, which have proved to be of great influence upon the characteristic taste of these cheeses. If the decomposition of the casein keeps within narrow limits, as in Gervais and in Edam cheese, the low figures for soluble and for amino

nitrogen demonstrate this just as clearly as do the mild tastes and flavors of these products. On the other hand, a high percentage of soluble and of ammonia nitrogen is characteristic of such cheeses as Limburger and Camembert, while a comparatively large percentage of amino nitrogen is indicative of the high quality of Emmental, Cheddar, Roquefort, Stilton, and similar cheeses. The following data gathered from analyses of ripe cheeses may illustrate these relations:

Kind of Cheese	Taste and Flavor	Per Cent of Total N Soluble	Per Cent of Soluble N	
			Amino-N	Ammonia-N
Gervais	Very mild	22	20	6
Edam		27	11	2
Emmental	Mild	33	52	7
Swedish hard cheese		38	60	7
Cheddar	Stronger	50	55	7
Roquefort		52	45	9
Camembert	Very strong	100	15	10
Limburger		99	5	12

These nitrogen figures vary greatly in each cheese with its age, and smaller variations are also noticeable in different samples of ripe cheeses of the same type. A fundamental difference exists between hard and soft cheeses insofar as in the first case the transformation of the casein proceeds uniformly throughout the whole curd, while in the second case the changes start at the surface and the white sour center remains unchanged for a long time. Here again it is demonstrated that anaerobic processes predominate in the hard, and aerobic ones in the soft cheeses. Naturally, taste and flavor are dependent not only on the transformation of casein, but also on the presence of other substances, especially of products of the decomposition of fat, and of salt. These substances, however, are to be found also in hard cheeses more frequently close to the surface, and because of this fact some authors have drawn the incorrect conclusion that aerobic organisms were of equal importance in hard as well as in soft cheeses.

Enzymatic and Bacterial Action upon Casein.—The ripening of sour milk cheeses shows most distinctly that microörganisms and their enzymes are of fundamental importance. Cheese prepared aseptically does not ripen at all, as was first demonstrated by Duclaux in France about fifty years ago. If rennet is used, the pepsin contained therein

participates in peptonizing the casein, provided the acidity is sufficiently high (above 0.3 per cent lactic acid), as is the case in Cheddar and most soft cheeses.

Peptonizing microorganisms and their enzymes are present in every milk; most important among them are the *micrococci* drawn from the udder. They become active in the cheese as soon as the lactic acid *streptococci* have produced enough acid. At the same time the acid produced activates the rennet pepsin, and the rennet in its turn stimulates the otherwise weak proteolytic actions of the streptococci, as has been discovered recently.[1] The formation of the relatively large quantities of amino acids present in hard cheeses, is largely the work of *lactobacilli,* while in soft cheeses various *fungi,* molds as well as yeasts, are most active in the dissolution and transformation of the casein. Symbiotic and antagonistic actions between different groups of microorganisms and their enzymes are of great importance in these complicated processes. Every type of cheese shows its own peculiarities, and it is easily understood that the investigations made during the last twenty or thirty years have by no means solved all the problems, although in general the whole situation is now well cleared. A Swiss bacteriologist, E. von Freudenreich of Bern, has made most valuable contributions in this respect. He has especially shown that the high quality of hard cheeses depends mostly on the work of lactobacilli, while aerobic sporulating bacilli are of no, or of subordinate, importance, although E. Duclaux had thought them to be most active and had called them, therefore, *Tyrothrix,* that is, cheese-thread. Strictly anaerobic spore-formers are, as a rule, equally unimportant; but they are very active in a few little known European cheeses of inferior quality, and sometimes they cause undesirable changes in other cheeses.

Microflora of Different Kinds of Cheese.—Peptonizing micrococci and lactic acid streptococci are present in every cheese, while the other microorganisms participating in the transformation of the casein differ greatly with the various types of cheese.

Hard cheeses of fine taste and flavor, such as Cheddar, Edam, Emmental or Swiss cheese, and Swedish hard cheese, are all rich in lactobacilli.

The various cheeses of Roquefort type, such as Stilton, Wensleydale, blue-Dorset, and Gorgonzola, contain also numerous lactobacilli, but the growth of a bluish-greenish mold, *Penicillium Roqueforti,* within the curd is most characteristic. This fungus is added in the form of moldy bread when Roquefort cheese is formed. The ripening cheese

[1] Chr. Barthel und E. Sandberg, *Centralbl. f. Bakt.*, II. Abt., vol. 49, 1919, p. 392.

is pricked with needle-like instruments in order to assure a sufficient growth of the aerobic fungus in the inner parts of the loaves.

The flat soft cheeses, such as Brie and Camembert, are covered by different molds; white Penicillium-species and varieties of Oidium lactis are most numerous among them. The gradual advance of their proteolytic enzymes from the surface toward the center can easily be ascertained by examining cuts of cheeses of different age. Aerobic bacteria take part in this process; upon ripe cheeses they appear as a characteristic reddish coating. If it is customary to use such soft cheeses while they are very young, as is done, for example, with Gervais and Neufchâtel cheese, the mold growth does not become very conspicuous, although its effect is quite noticeable if these cheeses are kept for a few days.

In another type of soft cheeses the mold growth is artificially suppressed by a special treatment, which only permits the development of certain proteolytic bacteria upon the surface, where they grow in a thick slimy layer. Their metabolic products are partly the same as in typical putrefaction (putrescin, cadaverin, indol, and hydrogen sulfide); accordingly, much depends on the personal disposition whether or not such a cheese, like Limburger, is accepted as human food. Very interesting data have been obtained concerning the symbiotic action of the bacteria growing in this cheese, which clearly demonstrate that results may be rather misleading if pure cultures only are tested. When the two species which are most characteristic of this cheese, were grown in milk, separately or combined, the following changes in the distribution of nitrogen were observed:

Increase or Decrease in Per Cent of Total Nitrogen	Soluble Nitrogen	Amino Nitrogen	Ammonia Nitrogen
Pure culture of B. casei limburgensis	+ 1.26	− 1.50	+1.13
Pure culture of M. casei liquefaciens	+51.06	+ 6.06	+1.13
Mixed culture of both organisms	+78.28	+31.88	+9.30

Transformation of Fat.—Much greater differences in regard to the transformation of fat than in the transformation of milk sugar and of casein are noticeable with the various types of cheeses. Since cheeses are made from cream, from whole milk, or from skim milk, their original fat content may vary widely. Furthermore, in certain cases the growth of aerobic organisms, especially of strongly fat-splitting molds, is favored by the form of the cheeses and the technique applied, as in Brie, Camembert, and Roquefort cheeses, while in other cases prac-

tically all surface growth is suppressed by rubbing the rind with salt or by covering it with paraffin. Because the splitting of fat is almost exclusively the work of aerobic bacteria and fungi, it proceeds most vigorously in soft cheeses of high fat content. If such cheeses become too ripe, their flavor turns distinctly rancid, as may be noticed especially with Roquefort cheese during summer. In hard cheeses more of the liberated fatty acids are found close to the surface, while flat soft cheeses may contain more of them in the center, due to evaporation and oxidation at the surface. The following figures illustrate these differences, giving the amounts of acids in gram per 1000 g. cheese:

		Butyric Acid	Caproic Acid
Emmental cheese	outer parts	1.232	0.928
	inner parts	0.176	0.116
Brie cheese	outer parts	0.466	0.128
	inner parts	0.572	0.139

The fatty acids are partly neutralized by ammonia. The glycerol is rapidly transformed, as is the case in rancid butter. Little fat is formed from casein by molds, but contrary to an opinion held for some time by different authors this process plays no important part in ripening cheese.

Taste and Flavor of Cheese.—Not all of the substances which in their entirety are responsible for the specific taste and flavor of the different kinds of cheese are known at present, but it is beyond dispute that all of them are regular products of the transformation of milk sugar, casein, fat, and of their derivatives. Formerly, much interest centered upon the discovery of aroma producing organisms in cheese as well as in butter, and it is, indeed, not very difficult to isolate from cheese different strains of bacteria and fungi which are characterized by their abilities to produce peculiar flavors, even when grown on the substrates commonly used in the laboratory. However, these characters are very easily lost; they are not species marks, but merely peculiarities temporarily acquired under the influence of prevailing environmental conditions.

The refreshing taste of young cheeses, such as Neufchâtel or Gervais, is mostly due to lactic acid, acetic acid, acetone, alcohol, and various esters. The taste of hard cheeses, such as Cheddar and Emmental cheese, is largely dependent upon the quantities of alanin, glycin, and other amino acids present. Certain strains of lactic acid

and of propionic acid bacteria have been found to act favorably upon the flavors of these cheeses. Taste and flavor of Roquefort and of similar cheeses are greatly influenced by substances produced by Penicillium Roqueforti in the transformation of fats. Free fatty acids contribute to the pungent flavor of ripe soft cheeses, and the ammonium salts of caproic, caprylic and caprinic acids are in the first place responsible for the peculiar "cheesy" odor of such products.

Texture of Cheese; Eye Formation.—Preparation and manipulation of the curd, forming, pressing, and further treatment of the young cheeses are largely responsible for the initial differences in the structure, which in their turn regulate the chemical processes that lead to more or less far-reaching changes of the texture in the ripening cheese. In soft cheeses the white loose acid curd is gradually transformed into a smooth, gelatinous, semi-transparent paste, while in hard cheeses these alterations are much less pronounced, due to the restricted acid formation and the less intense dissolving of the casein.

The firmer and more plastic texture of the hard cheeses favors the appearance of uniformly distributed openings in the curd, usually called *eyes*. Gases, mostly carbon dioxide, liberated in the fermentative changes of lactates and of glycerol, accumulate at spots where drops of whey remained inclosed in the curd. This eye formation is especially characteristic in Emmental cheese, and no cheese of this type is accepted as of first quality, if it is not "well opened," that is, if it does not have a sufficient number of very regularly formed and uniformly distributed eyes, each of about 1 cm. diameter. The gases which form these eyes are liberated in the transformation of lactates to propionates. Because this process is of minor importance, it is easily understood why occasionally "blind" Emmental cheeses are found which are also of good taste and flavor. In the manufacture of other hard cheeses much less attention is paid to this eye formation; in fact, it is almost entirely absent in many cases. The normal "opening" of the cheeses should not be confounded with "gassiness," that is an abnormal and excessive production of gas by organisms of fecal origin.

Abnormal Alterations.—If the transformations of milk sugar, of casein, and of fat do not proceed simultaneously in a well balanced manner as in normal cheese, the result will be unsatisfactory in one or another direction. Excessive acid formation makes an unripe sour product; predominance of casein decomposition causes a disagreeable bitter taste and putrid odor; too far-reaching splitting of the fats gives a distinctly rancid cheese. However, abnormal tastes and flavors may also be produced by certain strains of microorganisms which do not belong to the regular microflora of cheese. Yeasts have repeatedly

been found to aet unfavorably, espeeially in Cheddar cheese, among the bacteria those of fecal origin are least desirable. Cheese poisoning is partly due to the presenee of large numbers of such organisms (B. coli and its relatives), but B. botulinus has also been found oecasionally. As said before, overripe cheeses contain substances of typically putrid character; it is very probable that in many cases where toxie effects have been observed, such cheese had been eaten.

Abnormal milk, such as eolostrum, or as is obtained toward the end of laetation, is not suited for the manufacture of high grade cheese. The regular examination of milk and of rennet in the fermentation test (see Plate X) is the most effective means of diseovering faulty material that might cause abnormal alterations in the eheese. The

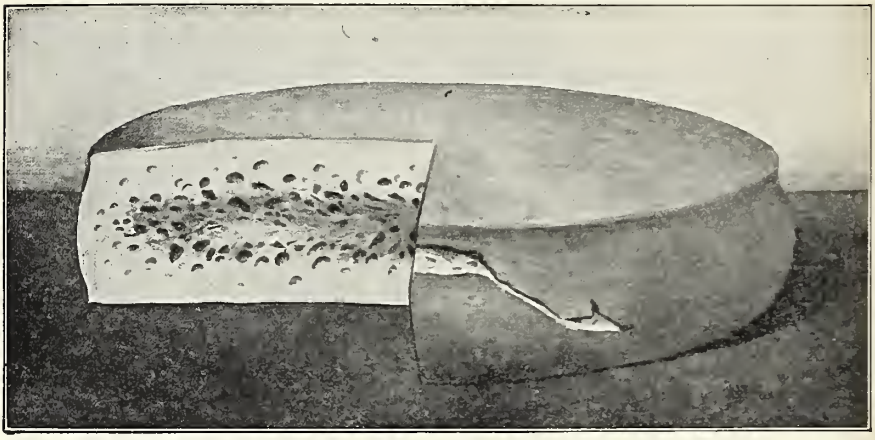


Fig. 49.—Blown Swiss cheese ($\frac{1}{10}$ nat. size).

formation of a smooth curd with none or only a few holes promises a satisfactory outeome, while a blown or torn eurd serves as a warning that failures will result, unless they are promptly checked by the application of preventive methods.

Gassiness; Blown Cheese.—Exeessive gas formation oeeurs if milk from inflamed udders is used, or if milk or rennet are heavily contaminated by fecal organisms (B. eoli and aerogenes, or B. amylobacter). Not all mastitis streptoeoeci, but some of them, produce considerable quantities of earbon dioxide from milk sugar, and mastitis milk itself is usually changed to such an extent in its chemieal composition that it disturbs the regular course of cheese ripening. B. coli and aerogenes liberate large quantities of carbon dioxide and of hydrogen from laetose during the first few days, sometimes even while the

cheese is still in the press. Fig. 49 shows a blown Swiss cheese that was cracked by the excessive gas formation. The discoloration of its torn center indicates that other unfavorable alterations occur in such cheeses. In fact, it is more on account of the disagreeable taste and flavor of blown cheeses than because of the change in texture that their market value is very much reduced. If anaerobic bacilli (B. amylobacter) get into the cheese in large numbers, the gas formation does not become noticeable before several weeks have elapsed, because the carbon dioxide and hydrogen liberated by them is derived from the lactates which they transform into butyrates. The latter again act unfavorably upon taste and flavor. Occasionally yeasts are responsible for gassy cheese, but in general they are much less detrimental than the two groups of bacteria named.

Blown cheese is not always characterized by very large and irregular holes. Sometimes the whole cheese becomes rather spongy, because the gases have made innumerable small holes, each of about 2 to 5 mm. diameter. Large holes are formed if the gas producing organisms have entered the curd as colonies attached to dirt particles, while small holes are the result of a more even distribution of single cells or of small conglomerates, as is regularly the case in milk that has passed through the centrifuge.

Abnormal Texture and Color of Cheese.—The disturbances in the structure of cheese caused by excessive gas formation leads, as a rule, to further deteriorations in its texture. Parts of the curd become abnormally hard, while others remain too soft and become more or less slimy. Similar alterations may be due to the presence of slime producing organisms, whose activity, however, is usually confined to the surface, where they are less detrimental than they would be, if they would also attack the inner parts.

Abnormal *pigmentation* of cheeses is only partly caused by microorganisms. If sour or well ripened milk is used, frequently small amounts of *metals* are transferred from the utensils to the cheese, where they are changed to sulfides which make the curd bluish, greenish, gray or black. If the cheeses are kept on boards made from spruce, the outer parts show often a reddish-brownish coloration which is caused by substances entering the cheeses from the wooden supports.

In other cases *bacteria and fungi* are the causes of abnormal yellow, greenish, bluish, reddish, brown, or black spots occurring upon or within the cheeses. Contrary to the diffused staining of non-bacterial origin, the pigment production by bacterial or fungous colonies is sharply localized. With proper technique, no great harm is to be expected from most of these organisms; only certain red, brown, and black varieties of lactic

acid and propionic acid bacteria may become rather troublesome, because as kinsmen of typical cheese bacteria, they find most suitable environmental conditions in every normal cheese. Accordingly, they must be traced to their source, and the initial infection must be prevented.

3. MEANS OF REGULATING THE ACTIVITY OF MICROORGANISMS IN CHEESE

The qualities of the different types of cheese are mainly influenced by the qualities of milk and of rennet, and by the technique applied in each case. It is very interesting to note how the bacteriological investigations made during the last decades have elucidated many of the methods, employed by experienced cheese makers, in regard to their inherent reasons, and that now the various technical modifications can be chosen and applied much more intelligently and successfully than at the time when all depended on experience and practical skill. The use of pasteurized milk and of pure culture starters has further stabilized the cheese industry, although much remains to be done in this respect until the whole problem will have been brought to an equally satisfactory status as has been reached in regard to cream ripening and butter making.

Influence of Milk and of Rennet.—The quality of the *milk* used is of importance chemically as well as biologically. Changes in its lactose content may lead to abnormally weak or abnormally strong acid formation with its harmful consequences. An unusually high fat content invites rancidity if soft cheeses are made. Very clean milk can be used successfully only if good starters are available, otherwise no clean and vigorous acid formation would be assured. The fermentation test will furnish the necessary information; a smooth curd with very little whey of clean taste and flavor is most desirable (see Plate X, Fig. 1*b*).

Rennet likewise acts chemically as well as biologically. Because of the pepsin contained therein a relatively large amount of rennet hastens the ripening process, at least in all cheeses of sufficiently high acidity. Rennet powders or tablets do not contain a valuable microflora; in connection with pasteurized milk normal cheese can be obtained only if good starters are used. In home-made rennet infusions, however, the useful lactobacilli are very numerous, provided that the stomachs have been carefully cleaned. If this was not done, or if the rennet infusion has been contaminated from other sources, gas producing organisms may predominate. Again the fermentation test will render very valuable service in the control of the rennet, together with determinations of its acidity.

Influence of Technique.—The extent to which the *milk* is ripened, that is, held for spontaneous souring, before it is coagulated, the curdling and the manner in which the curd is manipulated, determine in the first place the kind of microflora and its activity in the young cheese. If the *curd* is heated, it becomes harder; and the higher the temperature, the more organisms are eliminated, excepting the lactobacilli that do not suffer even if 55° C. are applied, as in the making of Swiss cheese. Yeasts are almost completely killed by this treatment, otherwise they might act very unfavorably as gas producers.

If the cheeses are *pressed*, their whey content is more or less reduced; accordingly, the curd becomes firmer, and the acid formation is reduced. It depends on the *form* and the *size* of the loaves whether the ripening will be more under the influence of aerobic or of anaerobic organisms, as was discussed before. *Temperature* and *humidity* of the curing and storage rooms affect the water content of the cheeses, and foster or check the development and activities of the various groups of microorganisms and of their enzymes. With Roquefort and American Cheddar cheeses very good results are obtained at comparatively very low temperature. Little harm can be done by the gas producing bacteria below 10° C.

The *salting* of the cheese acts differently according to its application. If the salt is added to the curd, but not evenly mixed with it, it will retard the acid formation in places; the dissolution of the casein may then prevail to such an extent that soft putrid spots will appear. If the salt is applied from the outside, it becomes possible to use this means for stimulating or retarding the biochemical transformations within the cheese. Because more salt accumulates close to the rind, it checks the action of susceptible organisms in this zone, as for instance that of the propionic acid bacteria in Swiss cheese; the characteristic eyes are absent within a few inches from the rind even in otherwise well opened cheeses. The bathing of Limburger in brine and the so-called "smearing" of its surface, which is kept moist continually, suppresses almost completely the growth of molds that is not desired in this cheese.

Special *treatment of the rind* helps to favor or to exclude aerobic organisms. Most effective in checking such growth is the application of a paraffin coating, as is now widely adopted for American Cheddar and for Swedish hard cheeses. It has proved equally useful for Edam and Roquefort cheeses, provided that in the latter case the inner parts are properly aerated by pricking. Placing the cheeses in hay or straw serves, on the other hand, as a rather crude inoculation of the rind with molds and other aerobic organisms.

In regard to *abnormal alterations* hardly anything can be done after

the cheese is made, except that by cooling (packing in ice) excessive gas formation can be checked. In all other respects "prevention is better than cure," and the fermentation tests permit an accurate control of milk, rennet, and water. Adding nitrate to milk (20 to 60 g. per 25 gallons) reduces to some extent the danger of getting blown cheeses. B. coli and aerogenes acquire the oxygen they need, first from the nitrate, which is transformed to ammonia; in the meanwhile the milk sugar is changed to lactic acid and no longer available to them. Butyric acid bacteria and yeasts, however, are not hindered by the addition of nitrate. Occasionally the saltpeter itself was found heavily infested by yeasts, and its use proved distinctly harmful.

If different kinds of cheeses are made side by side, or if the manufacture of one kind is replaced by that of another, it may happen that queer "*crosses*" are produced, as for instance Camembert with Limburger flavor. Local varieties, as are characteristic of the one type of cheese, are liable to continue their growth and activity for a while in the other cheese despite the changed technique. Therefore, great care is necessary if cheeses of different kind are to be made simultaneously. Separate sets of utensils and separate rooms are necessary for obtaining faultless products. General disinfection and introduction of the desired organisms must accompany the change from one to another type of cheese making.

Use of Pasteurized Milk and of Starters.—It would be best if for cheese making as for butter making pasteurized milk and selected starters could be used generally. At present, however, this goal has not been reached. With soft cheeses the least difficulties are encountered. Heated milk gives a soft curd which offers no disadvantage in such cases, and the active microorganisms are well known, therefore, first-class starters can be prepared. Hard cheeses offer greater difficulties, mainly because of the abnormal consistency of the curd. That excellent hard cheeses can be made from milk pasteurized at high temperatures, was first proven by Danish and Swedish cheese-makers. But because the chances are very slight that pathogenic organisms are carried by hard cheeses, the use of clean raw milk is equally satisfactory or even preferable, because of the firmer texture of the curd. This point is of greatest importance in the manufacture of Swiss cheese.

The low germ content of pasteurized and of clean raw milk necessitates, of course, the use of starters. Sour milk, buttermilk, and whey have served as such for a considerable length of time. Carefully prepared rennet infusions contain all the bacteria needed for Swiss and other hard cheeses. Moldy bread has long been used for inoculating Roquefort cheese; in Stilton cheese the fungi were transplanted by inserting

plugs taken from old cheeses. Quite generally a heavy inoculation of the milk with the desired microorganisms proves very useful; it can easily be made by adding to the milk an emulsion prepared from a piece of first-grade half ripe cheese of the type to be made.

Pure Cultures for Cheese Ripening.—Extensive and successful tests with selected pure cultures have first been made in the manufacture of Swiss cheese by E. von Freudenreich at the Experimental Station Bern-Liebefeld, Switzerland. Lactobacilli of different types have been

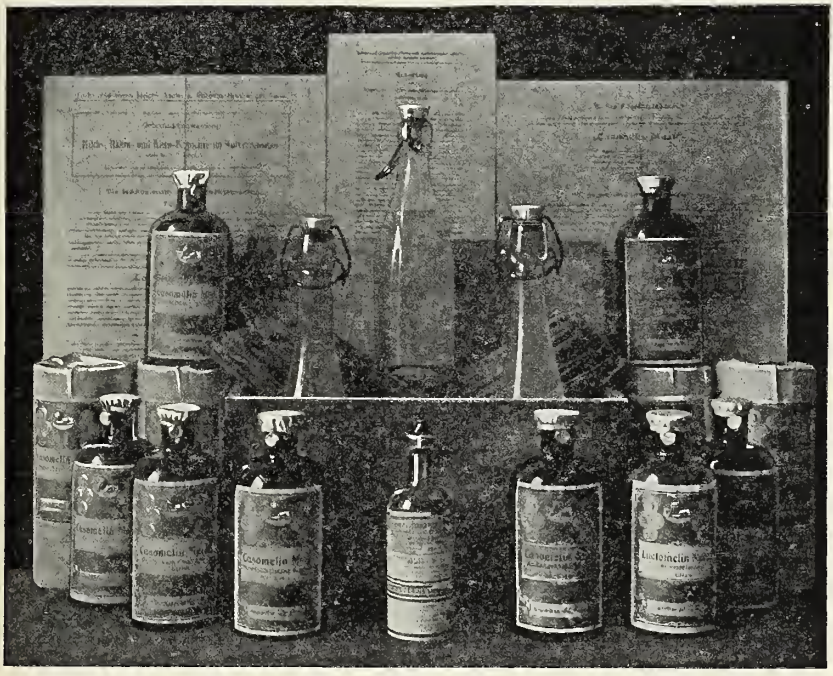


FIG. 50.—French, Swiss and Italian cultures for cheese ripening ($\frac{1}{5}$ nat. size).

used; one called *B. casei* ϵ was found to be most useful, especially when combined with pure cultures of propionic acid bacteria. Such cultures are now being supplied by various institutions in Europe; in the United States the Dairy Division of the Federal Department of Agriculture has made successful experiments, and cultures may be obtained from there.

For Cheddar cheese pure cultures of various types of lactic acid streptococci as well as of lactobacilli are used; the latter give a more highly flavored product, as was to be expected.[1]

[1] W. STEVENSON, *Trans. Highland and Agric. Society of Scotland*, 5. Ser., vol. 30, 1918, pp. 97–125.

For the various kinds of French soft cheeses, such as Brie, Camembert, Coulommiers, and Roquefort, selected cultures are now being supplied in liquid and in powder form by the Institut Pasteur of Paris and by several commercial firms.

In Italy an "Associazione pro Grana" furnishes cultures for the hard Italian cheese, so-called Parmesan or Grana.

Some of the cultures as they are supplied by experimental stations and by the trade, are shown in Fig. 50.

CHAPTER XII

SEWAGE DISPOSAL

A never failing supply of clean water is of fundamental importance on the farm. The quality of milk and of dairy products depends very much on the purity of the water, as was repeatedly pointed out on the preceding pages, and the same holds true in regard to the general health of man and animal. Unfortunately, pollution of well and spring water is by no means rare. Carelessness in disposing of the discharges from the farm is much too wide-spread. Wherever running water is available it is frequently used to carry the wastes away, often without giving adequate consideration to the inconvenience and possible danger such practice may involve. Where running water is scarce, careless disposal of human excrements and of other waste products does not only constitute a serious nuisance, but again it may become dangerous, because at such places flies are exceedingly numerous which act as carriers of many harmful, and perhaps pathogenic microorganisms. Proper disposal of farm as well as of city sewage implies a multitude of economic and of engineering problems, which can be correctly solved only if the pertinent bacteriological facts are fully considered.

Methods of Sewage Disposal.—Sewage is a very variable mixture of soluble and insoluble waste products of different origin and of unequal composition. All these substances are ultimately dissolved and transformed into mineral matter by bacterial action, but the time consumed for completing these transformations may be short or long according to circumstances. Highly diluted solutions, such as discharges from washbasins, bathtubs, floor drains, etc., contain very little or no foul matter, and they may be carried away by running water without disadvantage. Human excrements, on the other hand, as well as other discharges rich in organic substances, as for instance dairy wastes, should be kept separate and treated in such a manner as to insure rapid mineralization of the organic residues and certain destruction of all pathogenic organisms that may be present. Saving of the plant food which these substances contain is of considerable importance, although frequently this point is treated rather

lightly. If no complete sewerage system can be installed, the collection of the human excrements in properly constructed and well kept privies deserves full consideration. If fibrous peat or ashes are easily obtained they may be used for converting the fecal substances into a dry earth-like manure, which however should be kept away from vegetables and berries, because of possible contamination. Peat closets are widely used in Europe; if cases of typhoid fever or dysentery occur, thorough disinfection can be accomplished without great difficulty.[1] However, unless the condition of a privy receives constant personal attention and care, it is always liable to become a nuisance and a source of danger to the health of man and animal.

The liquid wastes from a pressure water system can not always be carried away by running water, and their disposal in an ordinary seeping cesspool, though still widely practiced, is strongly to be condemned. If excretal matter is flushed away and mixed with other liquid wastes, such sewage should always be prevented from entering the soil before it has been properly treated to make it as inoffensive and harmless as possible. Fresh sewage is much inclined to undergo anaerobic decomposition, and the best place for this is in a properly constructed watertight septic tank. After the destruction of organic matter has proceeded therein to a point where the cooperation of aerobic bacteria is needed to complete the mineralization, the sewage may be used for irrigation, or it may be oxidized upon so-called trickling filters, or it may be discharged into a river.

The loss of plant food makes the last-named arrangement a practice which is permissible only so long as the land itself is productive and the population not very dense. Eventually the conservation of all plant food becomes more and more imperative; but since the simple and careful, though unhygienic, handling of all waste products as it has been practiced in China through many centuries, would not find favor with any Western nation, other methods must be found which retain the advantages of the modern sewage treatment, but are equally satisfactory from the viewpoint of conservation of plant food. The so-called activated sludge process, recently introduced in American and British cities, offers good prospects in this direction. A carefully installed sewerage system is not too expensive for the farm, although the opposite opinion is frequently held.[2]

[1] *Arbeiten der Deutschen Landwirtschafts-Gesellschaft*, Heft 1; A. Stutzer und E. Herfeldt, *Centralbl. f. Bakt.*, II. Abt., vol. 1, 1895, p. 841.

[2] For detailed and illustrated descriptions of the various methods of sewage disposal see U. S. Public Health Service *Bull.* 101, 1919, and U. S. Dept. Agr. *Farmers' Bull.* 1227, 1922.

Septic Tanks.—All sewage should be removed from the farm buildings through a water-tight sewer to a water-tight tank, where it may undergo sedimentation and fermentation. Contamination of the water supply may take place if foul matter is allowed to drain off into the ground from leaking pipes or from an open cesspool. Shallow wells are not safe unless they are located more than 200 to 500 yards from privies, stables, or other sources of pollution. Only where the water has to pass through a vertical column of soil of more than 20 feet depth, is the complete removal of the bacteria to be expected. Since the movement of the bacteria-laden liquids is mostly down-hill, following the slope of the land, it is very desirable to have the well always at a higher level.

Within the septic tank all heavy insoluble substances settle down

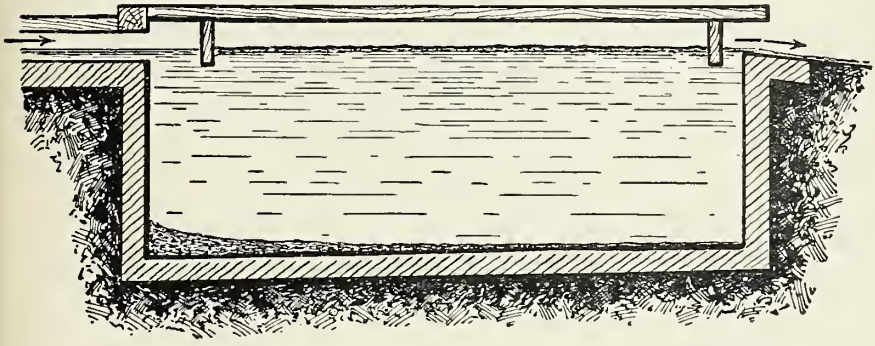


Fig. 51.—Septic tank, after Kolkwitz.

as sludge, while the lighter particles, including all greasy material, accumulate on the surface as a scum, partly carried by the gases which are liberated in the fermentative processes. Because of the anaerobic conditions methane is produced in the largest quantities; the rest of the gaseous mixture is made up of hydrogen, nitrogen, and carbon dioxide. Figure 51 shows a septic tank of simple construction with its sludge deposit at the bottom and the greasy scum at the surface, which is prevented from escaping with the effluent by boards attached to the cover. Naturally, the activities of the anaerobic bacteria in breaking down and dissolving proteins and other organic substances will always give rise to such offensive odors that it is far better to have the septic tank not only sufficiently far away, but also tightly covered. Its effluent which still contains large quantities of partly dissolved organic matter, should be carried away underground to a place where final oxidation will be effected. Since the activities of

the bacteria are disturbed in the ordinary tank by the inflow of fresh sewage, it is preferable to have two chambers, the first one serving as a settling chamber and the second one as a fermentation chamber, affording most suitable conditions for vigorous development and activity of the anaerobic bacteria. Grease traps, sludge drains, and siphons for removing the effluent are important practical improvements.[1]

Irrigation.—If the effluent of the septic tank is used for irrigation the aerobic organisms of the soil will quickly destroy all organic residues and mineralize the plant food contained therein. Surface

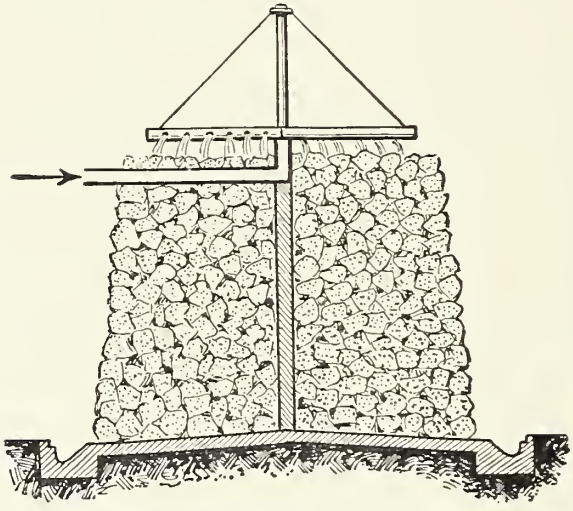


FIG. 52.—Trickling filter, after Kolkwitz.

irrigation, as frequently practiced in Europe, has great disadvantages especially during winter; subirrigation or underground irrigation is far superior. For this purpose the water from the septic tank is distributed by a system of drains placed about one foot below the surface in porous ground to attain proper aeration and also sufficient protection against frost, which can be increased, if necessary, by covering the run in cold weather with straw, hay, or leaves. Permanent grass land is generally best suited for subirrigation. Deep underdrainage is necessary, and any well or spring used for water supply should be located not less than 100 yards uphill.

[1] For details of constructing and operating septic tanks see U. S. Dept. of Agr. *Farmers' Bull.* 1227.

Trickling Filters.—Complete mineralization of all organic substances by aerobic bacteria can also be attained by passing the water through a porous filter similar to that shown in Fig. 52. It may be constructed of coke, gravel, broken stone, or of other material, and the sewage may be applied either continuously by means of a sprinkler, or intermittently in so-called contact beds. The sprinkler system offers maximum aeration, but good results may be obtained also with the intermittent system. While the filter is filled for a few hours absorptive processes take place which are followed by vigorous oxidation, as soon as the liquid is drained off. Comparative tests made with dairy wastes showed, for instance, the following oxidation of organic substances in per cent of what had been added:[1]

	Organic Matter	Organic Nitrogen	Fat	Milk Sugar
	Per Cent	Per Cent	Per Cent	Per Cent
Sprinkler system	73–87	66–86	66–98	100
Contact bed	47–62	45–62	87–95	85–100

However, good results are assured only if the filters are carefully handled and controlled. By testing sewage and effluent with potassium permanganate the degree of oxidation can be easily ascertained. In the experiments just mentioned it was 93 to 98 in the first, and 58 to 93 per cent in the second case. Under ordinary farm conditions subirrigation is undoubtedly to be preferred; while for the disposal of dairy wastes artificial filters may prove very helpful. They must be covered to prevent odors and exclude flies, and in cold weather they must be warmed to 15 to 30° C., so that the bacterial action can proceed without interruption.

Activated Sludge.—The so-called activated sludge process seems to offer good prospects to the cities as an efficient means of sewage disposal, and to the farmer as a valuable method of regaining the plant food that is brought daily from the farm to the city in form of human food. The activated sludge produced contains about 5 per cent nitrogen, of which $^1/_2$ to $^2/_3$ was found to be easily available when tested in fertilizer experiments.[2]

The underlying principle of the activated sludge process consists

[1] A. Kattein und F. Schoofs, *Milchzeitg.*, vol. 32, 1903, pp. 98, 114.

[2] *Report* of the Rothamsted Experiment Station, 1918–20, p. 56; *Third Ann. Rep.* Sewerage Comm., Milwaukee, Wis.

in replacing the anaerobic fermentations in the ordinary tank by aerobic transformations. For this purpose the sewage is supplied with large quantities of aerobic bacteria (in activated sludge) and with compressed air to insure vigorous action of these organisms. The sewage circulates for several hours in long or circular tanks, and the brown jelly-like mass which settles down, is dehydrated and ground. No offensive odors are produced and the effluent is nearly potable. The costs are high, but they are partly covered by the revenues from the fertilizer sale. Not much space is needed, and in this as in other respects the activated sludge process recommends itself as an almost ideal method of city sewage disposal.

Chemical Treatment.—No complete purification of sewage is possible by chemical means, but such substances may be used advantageously for accelerating the settling of the sludge in the tank and for sterilizing the effluent before it is discharged into a stream. Milk of lime, alum, iron sulfate, and sodium silicate may serve as clarifiers in settling tanks; chlorination or ozonization of the effluents are the most effective means of eliminating any possible danger that might be expected from the presence of pathogenic organisms. If fecal masses are to be sterilized by chemicals, as in cases of typhoid fever, very large quantities are needed, otherwise the disinfection will remain incomplete.

CHAPTER XIII

BACTERIA AND RELATED MICROORGANISMS IN BARNYARD MANURES

The gradual reduction in the natural fertility of all agricultural soils necessitates the application of farm manures and of artificial fertilizers. The latter have been known for little more than one hundred years, while barnyard and green manures have been used for thousands of years in Europe as well as in Asia. At present the application of artificial fertilizers is of very great importance for European agriculture, because the very dense population requires so much food that the productivity of the soils must be increased to the utmost. However, the use of farm manures has not lost its fundamental importance, and if all the plant food contained in human, animal, and plant residues is carefully collected and given back to the soil, enough food can be produced without the use of artificial fertilizers even in such densely populated countries as in China. There is no doubt that in America the natural fertility of the soils could be retained or restored to a large extent solely by the use of farm manures; but widespread carelessness in the handling of manure causes enormous losses in plant food which amount to several hundred million dollars annually. It is true that the high cost of labor frequently militates against the extensive use of bulky farm manures, and in such cases the application of artificial fertilizers is no doubt preferable. On the other hand, it is an indisputable fact that great losses in humus content are the main cause of the decreased productivity of American soils, and humus can be restored only by the consistent use of farm manures, while artificial fertilizers exert hardly any beneficial effect in this respect. Animal manures are of special importance, because they not only contain large quantities of organic substances, but also large numbers of bacteria and related microorganisms, which participate in the humus formation and in other transformations of plant food in the soil.[1]

[1] For references see F. Löhnis, "Handbuch der landw. Bakteriologie," Chap. IV.

1. GERM CONTENT OF BARNYARD MANURES

The solid excrements of animals are made up of partly decomposed food residues and of the bacteria that cause their decomposition. Unstained smears give very interesting pictures like that shown in Fig. 53. The weight of living and of dead bacteria in the feces was found to be equal to $^1/_{10}$ to $^1/_5$ of the weight of the dung; calculated on the basis of fresh weight the number of living cells would approximate 20,000 to 40,000 millions per g. If plate cultures are made, usually no more than a few hundred millions per g. will grow, but such results are of restricted value, because many of the fecal bacteria refuse to grow on the plates. From human feces as many as 18,000 millions per g. have been cultivated. Fresh urine contains, as a rule, comparatively few microorganisms, and those present in the litter are also of minor importance, so far as numbers are concerned; but in regard to the activities performed by the different groups of microorganisms the bacteria growing in urine as well as those on the straw are as essential as those in the feces.

FIG. 53.—Smear made from cow dung, unstained (×700).

Frequency of Microorganisms in Manure.—The total germ content of barnyard manure varies widely according to the composition and the age of the material. Many bacteria are already present in fresh stable manure, but if this is kept for several weeks or months, at first a marked multiplication takes place which is followed by a gradual decline. Plate counts gave, for instance, the following results whose relations are of greater interest than the numbers themselves because of the reason discussed above. From fresh material were grown in millions per g.:[1]

Feces	Urine	Straw
390–480	1–2	1, 3–19

These materials were mixed as in average manure in a relation of 7 parts feces to $1^1/_4$ part urine to 1 part straw, and kept for six weeks at 20° C. Then the plate counts showed in millions per g.:

In Feces and Straw	In Feces, Urine, and Straw	In Urine Alone
4800–5700	11,000–11,600	3

As was to be expected, the bacterial growth was much more vigorous in the mixture made up of feces, urine, and straw, than in feces and

[1] F. LÖHNIS and J. H. SMITH, *Fühling's landw. Zeitg.*, vol. 63, 1914, p. 153.

straw without urine, or in urine alone. Nevertheless, feces and straw alone showed also a marked increase in numbers, which fact is of special importance because certain reasons make it desirable to keep solid and liquid manures separate as much as possible.

It may be assumed that 100 lbs. of ordinary barnyard manure contain approximately 1 to $1^1/_2$ lbs. of living bacteria and fungi. If 15 tons of manure are applied to one acre of land, no less than 300 to 450 lbs. of living matter are incorporated in the soil together with about 6000 lbs. of organic matter. It is easily understood that the biological conditions in the soil are greatly affected by such a treatment.

Groups of Microorganisms in Manure.—The very complex mixture of all kinds of organic residues stimulates the development of practically all groups of microorganisms. The anaerobic B. putrificus and related forms work together with B. fluorescens, proteus, and other aerobic species in dissolving and disintegrating proteins and other nitrogenous compounds. B. Pasteuri and many other rod-shaped as well as coccoid bacteria transform the urine nitrogen to ammonia. Anaerobic butyric acid bacilli, aerobic sporulating bacilli, B. coli, B. aerogenes, and various streptococci are breaking down the remaining soluble carbohydrates, while pectic substances and cellulose are attacked by special groups of organisms which are again partly anaerobic and partly aerobic. Denitrifying bacteria are frequent in all manures, whereas nitrifying organisms are absent in fresh and not very numerous in old manure. Anaerobic and aerobic nitrogen fixing bacteria are equally present. The Actinomycetes constitute another characteristic group of manure organisms, and the same is true of yeasts and molds. In dry manure heavy mold growth is always visible with the naked eye. Higher fungi, too, grow well on rotted manure, as is demonstrated by artificial mushroom cultures. Protozoa are frequent, and some other interesting groups, the Myxomycetes and the Myxobacteria, also grow well on manure. The latter are characterized by a curious mode of colony formation and of fructification,[1] which make them of great systematic and physiological interest; but they are of minor importance as far as the biochemical transformations in the manure are concerned.

If pathogenic organisms get into the manure, as may easily happen in cases of foot and mouth disease, mastitis, and tuberculosis, they may remain alive and may even multiply to some extent, and such manure is very liable to become a source of new infection. It is possible to destroy these pathogenic organisms in the manure by piling it in

[1] R. Thaxter, *Botan. Gazette*, vol. 17, 1892, p. 389; vol. 23, 1897, p. 395; vol. 37, 1904, p. 405. Striking colored pictures were published by Quehl in *Centralbl. f. Bakt.*, II. Abt., vol. 16, 1906, pp. 9–34.

loose high heaps wherein the temperature quickly rises to about 70° C.,[1] but under ordinary farm conditions such a treatment would be of doubtful value; if the highly resistant spores of the anthrax bacillus are present, it would be quite insufficient. Thorough disinfection with strong acids, or the destruction of infected manure by fire are the only reliable means of rendering such material harmless.

2. BACTERIAL ACTIVITIES IN BARNYARD MANURES

It is a well known fact that the chemical composition of animal manures shows wide variations, and still more irregular are the effects realized from the application of barnyard manures. In field experiments of four years duration the following quantities of nitrogen, phosphorus, and of potassium were recovered in the manured crops:[2]

Nitrogen	Phosphorus	Potassium
Per Cent	Per Cent	Per Cent
7.8—46.1	10.1—75.6	22.4—85.1

Similar results have been obtained quite generally, but, as a rule, no investigations have been made to find out why the effects did show such wide variations. Since the manure produced annually represents an economic value of approximately $50 per full grown animal, it is rather disappointing that so little has been done thus far to discover the chemical and biological reasons for these wide differences. It is beyond doubt that after such investigations will have been made, and the conditions are known under which a high fertilizing effect is assured, the intelligent application of barnyard manure will prove much more profitable than is frequently the case to-day.

The Rotting of Manure.—There is a rather widespread belief that it is best to apply the manure as soon as it is made; losses which may occur during storage are thus excluded, and all plant food is given back to the soil. However, this practice gives satisfactory results only if sufficient time, at least 6 to 8 weeks, will elapse before a new crop is planted on the manured field. Otherwise the undecomposed organic substances will prove distinctly harmful to the crop, because they stimulate the bacterial assimilation of nitrate, ammonia, and amino nitrogen in the soil, and thereby cause a nitrogen starvation of the higher plants. For example, the following percentage of manure nitrogen was taken up by four successive crops, when the same mixtures that were used for making the bacterial counts mentioned above,

[1] H. Bohtz, *Arbeiten a. d. Kais. Gesundh. Amte*, vol. 33, 1910, p. 313.
[2] *Arbeiten d. Deutschen Landw.-Gesellschaft*, Heft 198, 1911.

were applied fresh or after they had undergone a rotting of six weeks duration:[1]

Per Cent N Recovered	Feces and Straw	Feces, Urine, and Straw	Urine Alone
Fresh manure	− 3.2	+14.7	+56.7
Six weeks old	+17.3	+40.1	+73.5

Partial decomposition of the organic substances proved highly beneficial in every case, and special tests confirmed that the crops were much better supplied with nitrate if manure six weeks old was used.

Great numbers of analogous observations have been recorded in the literature. Two months rotting permits the removal of harmful carbonaceous substances and the initial decomposition of part of the nitrogenous compounds; immediately after such manure has been plowed under nitrification sets in. Nevertheless, the labor problem and the certainty that large losses in plant food will occur if the manure is not stored and handled very carefully, may make it advisable to apply the manure as soon as possible after it is made. If the new crop is not planted before 2 or 3 months have elapsed, and if the temperature is not too low, the necessary transformations will take place in the soil itself. Some weeks of "ripening" before application also increases the beneficial effect of liquid manure; if used fresh, "burning" of the plants is often noticeable.

Transformation of Carbohydrates.—A loss of 10 to 30 per cent of the total solids is always to be expected during the rotting of manure. It is mainly caused by the fermentation of carbohydrates. If manure is carelessly stored or kept for a very long time, the losses may reach 50 to 70 per cent of the total dry weight. The percentage of soluble carbohydrates is lowest as far as the composition of fresh manure is concerned, but in regard to the rate of decomposition they rank first. Next come the pectic substances, while the cellulose is relatively most resistant and present in largest quantities. Comparative tests have shown the following relations in the extent to which these three groups of carbohydrates are being broken down during the normal rotting of manure. Of the total quantities originally present the following percentages underwent fermentation:

Soluble Carbohydrates	Pectic Substances	Cellulose
20 to 30 per cent	15 to 20 per cent	7 to 10 per cent

[1] F. Löhnis and J. H. Smith, l. c.

Because all these substances may be attacked by aerobic as well as by anaerobic bacteria, it does not make much difference whether the fermentations take place in the presence or in the absence of air. Under otherwise equal conditions the quantities transformed are approximately the same. But very great differences become apparent if samples of the same manure are allowed to rot at low or at high temperatures. Usually the losses in carbohydrates are 4- to 8-fold larger at 35° than at 15° C. Since heat is generated in the fermentative processes themselves, it is of great importance to keep the manure sufficiently moist and cool.

All kinds of organic acids are produced in the decomposition of

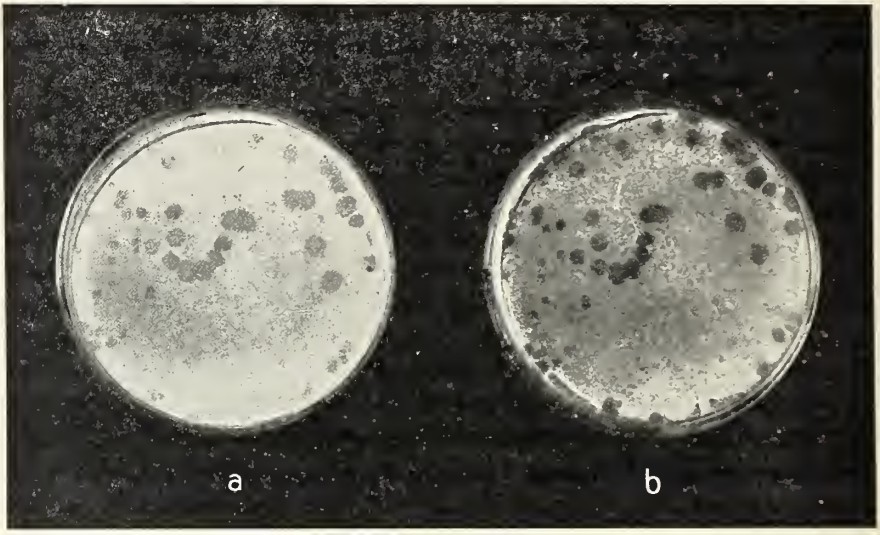


FIG. 54.—Cellulose agar culture ($\frac{2}{5}$ nat. size), (*a*) before, (*b*) after treatment with hydrochloric acid.

carbohydrates. Butyric acid plays an important rôle among them; it is partly responsible for the peculiar odor of rotted manure. If the ammonification remains low, the acid formation may be followed by the development of a distinctly acid reaction in the manure.

Frequency and Activity of Cellulose Bacteria.—There is little known at present about the actual numbers of aerobic and anaerobic cellulose destroying bacteria in manure. That they, too, multiply rapidly during the first few weeks has been ascertained by J. H. Smith,[1] who observed a ten-fold increase within six weeks. But more important

[1] F. LÖHNIS and J. H. SMITH, l. c.

than this increase in numbers of microorganisms is the fact that the active enzymes are soluble and exert a far-reaching effect even where the bacteria do not grow, or after they have died. In Fig. 54 a cellulose agar plate culture is shown in which very minute, nearly invisible colonies of aerobic cellulose bacteria are surrounded by comparatively large clear zones. Their appearance is similar to that produced by the growth of lactic acid bacteria in sugar agar containing chalk (Fig. 44, p. 177). However, it is not the dissolution of added chalk, but the disintegration of the cellulose present in the agar that causes the formation of these clear zones. When the plate was treated with hydrochloric acid all chalk was dissolved, but the clear areas were left unchanged.[1]

Gas Formation.—The gases liberated in the transformation of carbohydrates and of other organic substances are usually composed of approximately equal parts of carbon dioxide and methane. In general not much hydrogen is found, although it is produced in considerable quantities in the decomposition of carbohydrates by the fecal lactic acid bacteria (B. coli and B. aerogenes) and by the anaerobic butyric acid bacilli (B. amylobacter). The reason why it is so rare in the gases escaping from the manure is that in secondary reactions, taking place in the anaerobic transformations of carbohydrates and of fatty acids, methane producing bacteria are making use of the hydrogen. Even carbonates can serve as a source of methane in the presence of hydrogen.[2]

Since usually 20 per cent or more of the organic substances are destroyed in the rotting manure, it can be easily calculated that very large quantities of gases are produced. If it is assumed that 1 cubic meter of manure contains 250 kg. of water-free organic substances, and 20 per cent of them, that is, 50 kg. containing 25 kg. carbon, are destroyed, approximately 46 kg. carbon dioxide and 16.7 kg. methane would be produced, equal to $2 \times 23.5 = 47$ cubic meters of gas. In actual tests made the quantities measured were between 10 and 100 cubic meters of gases per 1 cubic meter of manure. Accordingly, all air spaces within a large manure pile are filled with carbon dioxide and methane. Such a condition is distinctly favorable to the activities of anaerobic organisms, and at the same time the evaporation of ammonia is materially reduced, because ammonium carbonate is much more stable in an atmosphere of carbon dioxide than in the presence of air.

[1] Microscopic pictures of such plates were published in a paper by F. Löhnis and Gr. Lochhead, *Centralbl. f. Bakt.*, II. Abt., vol. 37, 1913, p. 490.

[2] N. L. Söhngen, *Recueil des Travaux chimiques des Pays-Bas*, etc., 2. ser., vol. 14, 1910, p. 238.

Humus Formation.—The color of old manure shows that part of the organic compounds is transformed into brown or black soluble and insoluble substances, commonly called humus. Although much remains to be investigated, it is certain that only a restricted humification is desirable in manure. It is known from the great resistance of peat that humus produced under anaerobic conditions is of little value as a source of plant food, and the same is true of the black tough manure as it occurs in the bottom layers of big manure piles that have been kept for a long time. Such material is often plowed up, practically unchanged, after a full season in the soil. Ultimately the plant food inclosed in such peaty flakes will become available, but a prompt fertilizing effect can not be expected. Undoubtedly, the interaction between amino acids and carbohydrates, which was discussed on p. 128, plays a considerable rôle in the humus formation in manure as it does in soil. Free ammonia, produced in the mineralization of organic nitrogenous compounds, causes also a brown discoloration of the straw.

Production of Heat.—The rapid decomposition of carbonaceous compounds invariably causes a rise in temperature in the manure pile. If the material is loose and relatively dry, as in horse manure, mostly aerobic processes take place, and the production of heat is very pronounced. In hot frames practical application is made of this process. Under anaerobic conditions, as a rule, much less heat is generated. Complete oxidation of glucose to carbon dioxide and water liberates, for instance, sixteen times as many calories, as are produced in the anaerobic transformation to carbon dioxide and methane. However, barnyard manure is such a complex mixture that a direct application of simple physico-chemical relations can not be made. In fact, it has been repeatedly recorded that despite a stronger heat production the loss in organic substances was actually smaller than in another instance where the increase in temperature was less conspicuous. Relatively dry manure loosely stacked reaches almost regularly 65 to 70° C., while tightly packed material, containing approximately 80 per cent moisture, does not easily go above 40° C. Excessive heat production indicates large losses in valuable material in the manure pile as in the ensiled fodder; it may lead to the formation of coal-like substances that are of very little value. Thermophilic and thermogenic organisms are frequent in every manure, but they can not do much harm if the manure is kept tight and well moistened.

Transformation of Nitrogenous Compounds.—The majority of analyses made of manures show nothing but the amount of total nitrogen and perhaps its gradual changes. In view of the different origin and the complex nature of the constituents of barnyard manure,

very little insight can be gained from such analyses. In fact, not much is known concerning the nitrogen metabolism of animal manure, despite its very great economic value. The nitrogen present in solid excrements and in the litter (straw, peat, leaves, etc.) is incorporated mostly in complex, insoluble, protein-like compounds which offer great resistance to the attacks of bacteria and are, therefore, very slowly mineralized. In urine, on the other hand, practically all of the nitrogen is present in soluble form, as urea, hippuric acid, and to a small extent as uric and glycin-phenylacetic acids. All these amino compounds are more or less easily transformed to ammonia; but in the presence of large quantities of soluble carbonaceous substances the opposite process may predominate, that is, ammonia and amino nitrogen may be assimilated by microorganisms and transformed to resistant protein substances. To what extent the addition of urine may influence the nitrogen metabolism in manures may be seen from the following data, recorded by E. B. Vorhees and J. G. Lipman[1] with fresh and rotted manures:

Percentage of Total Nitrogen		Organic Nitrogen		Ammonia Nitrogen
		Insoluble	Soluble	
Feces and straw only	fresh	85.0—86.2	6.1— 7.4	7.6— 7.7
	rotted	76.8—83.2	7.9—10.1	8.9—13.1
Feces, straw, and urine	fresh	45.6—51.6	15.8—17.2	32.6—37.7
	rotted	62.4—67.1	8.2— 9.5	23.4—29.4

The solubility of the organic nitrogen showed an increase in the first, and a decrease in the second case; the same holds true in regard to ammonia nitrogen.

If samples of the same material are kept under aerobic and under anaerobic conditions, usually the insoluble organic nitrogen increases in the aerobic samples, and decreases in the anaerobic samples; the ammonia content rises more rapidly in the absence of air, and the losses in total nitrogen are much larger under aerobic conditions. Considering all these variable factors it is easily understood why the nitrogen contents of barnyard manures exhibit very great differences in quantity as well as in quality. The following data illustrate this point:

[1] *Annual Report* N. J. Agr. Exp. Stat., vol. 26, 1905, pp. 140, 161.

Percentage	Manures from		
	Horses	Cattle	Sheep
Total nitrogen	0.39—0.70	0.34—0.65	0.55—1.22
Amino nitrogen	0 —0.07	0 —0.08	0.03—0.14
Ammonia nitrogen	0.07—0.34	0.04—0.20	0.22—0-47

Close relations between ammonium content and fertilizing effect of stable manures have been observed frequently, though not regularly. This explains in part why either very satisfactory, or rather unsatisfactory results are obtained by the application of different farm manures, whose chemical composition, as a rule, is unknown.

Ammonification. Feces and straw show little ammonification; but this process is very strong in urine. Comparative tests with such material held for six weeks at 20° C. furnished the following results:[1]

	Feces and Straw	Feces, Straw, and Urine	Urine Alone
Per cent of total nitrogen transformed to ammonia...	2–2.8	28.4–30	75–80

Even if the tests were continued for a much longer time, not more than $^1/_5$ of the nitrogen in mixtures made up of feces and straw was transformed to ammonia. The following facts explain this high resistance. Dead and living bacteria constitute 10 to 20 per cent of the dry solids of feces, whose total nitrogen content, as a rule, was found to be 2 to 3 per cent, while dried bacteria contain usually about 10 per cent of nitrogen. Accordingly, approximately *one half of the total nitrogen* of the solid excrements is incorporated *in bacterial cells.* One half of the bacteria is alive, hence this nitrogen is not available. The other half is dead, but the mineralization of bacterial proteins proceeds rather slowly, as was pointed out before (p. 106). The nitrogen left in the indigested food residues is, of course, very resistant, otherwise the powerful digesting enzymes of the animal organism would have made use of it. Straw contains but little nitrogen, and not more than $^1/_4$ of it is digested by pepsin in the presence of hydrochloric acid.

[1] F. Löhnis and J. H. Smith, l. c.

Furthermore, its relatively high carbon content is not favorable for a vigorous ammonification.

The amino nitrogen in urine, on the other hand, is practically completely transformed to ammonia within a few weeks. If no loss occurs by evaporation, 80 to 90 per cent or more of the total nitrogen of well fermented liquid manure is present as ammonium carbonate, as organic ammonium salts, or as free ammonia. Because in the transformation of hippuric acid relatively large quantities of benzoic acid are split off (see p. 100), liquid manuring adds to every acre of land several hundred pounds of benzoic acid and a smaller quantity of phenols. It is obvious that the resulting change in the composition of the soil solution must exert a considerable influence upon the microflora of the soil.

Assimilation of Amino and Ammonia Nitrogen.—If urine is mixed with feces and straw a profound change in the nitrogen-carbon ratio takes place; ammonification is more or less checked, and in the presence of air the assimilation of ammonia and amino nitrogen is greatly stimulated. The analyses made by Vorhees and Lipman, quoted above, demonstrate these facts very clearly; ammonia and soluble organic nitrogen decrease in the complete mixture, while the insoluble nitrogen increases. Opposite relations prevail in the mixture free of urine. 30 to 70 per cent of the urine nitrogen may be assimilated if the liquid excreta are mixed with feces and straw. It was mentioned before that many bacteria and fungi are enabled to assimilate all soluble ammonia and amino nitrogen within a few days. These organisms are generally more active in the presence of air, but partial assimilation takes place also in the absence of air.

If peat is used instead of straw, more of the ammonia is retained, but some assimilation still occurs, and part of the ammonia escapes by evaporation. It is much better to collect and to use liquid manure separately from the mixture of solid excrements and straw. In liquid manure the ammonification proceeds undisturbed by those retrograde transformations, and the best manuring effect is assured. Wherever the cost of labor and other economic conditions permit, this system should be adopted.

Nitrification.—As a rule, direct nitrate determinations in manure give almost or completely negative results, but this does not prove that nitrification is entirely absent. Here the situation is similar to that noted with ammonia. As soon as ammonia is produced, it may evaporate or may be assimilated, and nitrite and nitrate may be destroyed by denitrifying bacteria or may be reduced and assimilated. The conditions under which vigorous nitrification takes place, are

(1) the presence of ammonium salts, but the absence of considerable quantities of free ammonia, (2) full aeration, (3) the absence of large quantities of soluble organic substances, (4) the presence of basic substances for neutralizing the acids. Urine alone, or mixed with feces and straw, tends to suppress the nitrification because of its relatively large content of soluble organic substances and of free ammonia. Feces and straw moistened with water offer more suitable conditions to the nitrifying organisms, which are, in fact, quite active in the surface layers of such manure in the pile, or after it has been spread on the field. If leaching is prevented a gradual increase in nitrate nitrogen may be observed, and even if the nitrate is washed away the nitrification is indicated by the multiplication of nitrifying organisms, which are always scarce in fresh manure.[1] Solid and liquid excrements are practically free from nitrite and nitrate bacteria, straw or other litter may contain few of them, but a regular source of infection is the old dirt present on stable floors, in the barnyard, etc. When tested in soil or in ammonium sulfate solution such material gives prompt nitrification.[2]

Losses of Nitrogen.—Nitrogen escapes from the manure pile into the air either as free ammonia or in elementary form. Losses of nonvolatile nitrogenous compounds by leaching should always be prevented, of course. A certain amount of ammonia is lost while the manure is still in the stable, because a heavy infection of the urine takes place as soon as this comes into contact with the floor, and the transformation of urea sets in almost immediately. These losses can be kept down by the use of peat; sawdust and straw, on the other hand, stimulate the evaporation because of their relatively large surfaces and low absorptive powers. In comparative tests, for instance, the following losses in per cent of the total nitrogen have been observed, when the manure was kept one day in the stable:[3]

Peat Litter	Sawdust	Straw Litter
7.1 per cent	11.1 per cent	19.8 per cent

Naturally, the evaporation of ammonia continues when the manure is removed from the stable. If it is spread on the field, further losses will be more or less completely prevented by the absorptive power of the soil, but in the manure pile the volatilization will continue

[1] B. Niklewski, *Centralbl. f. Bakt.*, II. Abt., vol. 26, 1910, pp. 388–442; E. J. Russell and E. H. Richards, *Jour. Agr. Science*, vol. 8, 1917, pp. 495–563.

[2] F. Löhnis and H. J. Smith, l. c.

[3] Hj. von Feilitzen, *Svenska Mosskulturfören. Tidskrift*, vol. 24, 1910, p. 10.

to reduce the ammonia content of the manure, especially if urine was mixed with feces and straw.

In addition to the escape of nitrogen in form of ammonia frequently large losses in elementary form have been observed. Their causes are not yet fully understood, but it is certain that such losses play a conspicuous rôle only in the presence of air.

Liberation of Nitrogen.—Denitrification is the only well-known process by which nitrogen may be liberated from the manure in elementary form. Since ammonia can not be nitrified in the absence of air, denitrification can be completely prevented if the air is excluded by placing the manure in tightly covered water-tight pits as soon as it is removed from the stable. If the manure pile is fully exposed to the air, as is usually done, nitrification will occur in the surface layers, and the nitrites and nitrates will be decomposed if they are either washed down into deeper layers where air is absent, or if anaerobic conditions are established by placing fresh layers of manure on top. Denitrifying bacteria and carbohydrates are present in abundance in every manure pile, and denitrification will always occur if nitrification is not prevented.

However, losses of nitrogen in elementary form have also been observed under conditions where nitrification could not be the indirect cause. The mechanism of these processes remains to be investigated; probably unstable compounds are formed in the decomposition of the organic nitrogenous compounds which break down to elementary nitrogen.[1] Whether or not free ammonia can be directly oxidized by bacteria to water and elementary nitrogen, as some authors have assumed, is another question that can not be answered at present. Altogether, it is much to be regretted that so little accurate work has been and is being done in regard to this very important problem. Year after year enormous economic losses continue to occur, and very little is done to prevent them.

Fixation of Nitrogen.—Occasionally instead of a loss, a gain in nitrogen has been found when the nitrogen balance was determined in samples of rotting manure.[2] Several nitrogen fixing bacteria have been isolated from such material. Most common, of course, are those belonging to the group of B. amylobacter; but Azotobacter, Bact. lactis viscosum, and other aerobic organisms capable of assimilating elementary nitrogen have also been discovered in manure. Because of their frequency in soil this is by no means surprising, and the large

[1] Russell and Richards, l. c.

[2] W. E. Tottingham, *Jour. Biol. Chem.*, vol. 24, 1916, p. 221; E. H. Richards, *Jour. Agr. Science*, vol. 8, 1917, p. 299.

quantities of carbonaceous material always present in manure make it probable that sometimes an increase in nitrogen may occur. However, these are rather rare exceptions; usually any gain that may take place is obliterated by large losses in nitrogen.

3. PREVENTION OF LOSSES OF PLANT FOOD FROM MANURES

Because the biochemical processes taking place in animal manure are incompletely known, it is impossible at the present time to decide definitely how the great losses of plant food can be prevented. But it is certain that they can be materially reduced if the necessary attention and care is given to this problem, and if proper use is made of the methods now available. The benefit which may be derived from such practice is not merely a personal matter for the farmer, but it is of still greater importance in regard to the conservation of national wealth. A loss of several hundred million dollars worth of plant food every year, solely because of improper handling of manure, is obviously no negligible fact. Mechanical, chemical, as well as biological methods may be used to reduce these losses. The first ones are of greatest importance, while the second and the third are playing subsidiary rôles.

Mechanical Methods.—Separate collection of solid and liquid manures is undoubtedly the most effective means of preventing large losses of plant food, of regulating the fermentative processes most advantageously, and of attaining the best possible fertilizing effects. Solid excrements and straw make the soil richer in bacteria and in humus; liquid manure, when carefully kept in air-tight and water-tight tanks, contains few bacteria but comparatively large quantities of available nitrogen and potassium. In different parts of continental Europe this method of handling animal manure has been practiced successfully for a long time. Certain practical difficulties exist, but they can be overcome, and they are greatly surpassed, as a rule, by the advantages of this method. The tanks for collecting and storing liquid manure must be, of course, water-tight and have a well fitting cover that prevents the escape of carbon dioxide and of ammonia. The outlet from the stable should not be above, but below the surface, to prevent disturbance within the fermenting liquid.

If feces, straw, and urine are mixed, the manure should be stored in water-tight covered pits. Fairly satisfactory results are assured if the manure is daily brought out from the stable, filled up in strips 6 to 8 feet deep, pressed down, moistened with water if necessary, and kept under cover.

Very valuable manure can also be secured by simply leaving it in

the stable on a water-tight floor, until it can be removed to the field. Labor and expense connected with the preceding methods are mostly saved; and because the desired fermentative processes are favored by a tight and moist condition of the manure pile, a well rotted material of comparatively high fertilizing effect will result. However, hygienic reasons are adverse to this arrangement; the shelter of the farm animals should not be at the same time the manure pile. The air is usually bad in such stables, and contagious diseases, such as tuberculosis and mastitis, may easily be carried to every animal in the stable.

Chemical Methods.—Since the middle of last century many tests have been made to find a simple and efficient chemical method for preventing all losses of plant food from manure. The results have been disappointing, because the fermentative processes are far too complicated and too little known to permit of an intelligent control by chemical means. Gypsum, rock phosphates, potassium salts, iron sulfate, and other substances have been found to be of little or no value. Because the breaking down of the organic substances and the multiplication of the bacteria is desired, these processes should not be disturbed by such admixtures. The use of acid phosphate can be recommended, because it exerts no detrimental effects, fixes part of the ammonia, and at the same time increases the phosphoric acid content which is always low in animal manure.

If the liquid manure is kept separate, practically all losses of nitrogen can be prevented by a careful use of sulfuric acid, of acid sodium sulfate, or of formaldehyd. The ammonium carbonate first formed easily breaks up to carbon dioxide and free ammonia; sulfuric acid and acid sulfate transform it to the non-volatile ammonium sulfate, while formaldehyd changes it to hexa-methylene-tetramin, which is readily nitrified in the soil. In careless hands concentrated sulfuric acid is not without danger, therefore, acid sodium sulfate deserves preference; but this, as well as formaldehyd, should not be added before the fermentation of the urine nitrogen is complete.

Biological Methods.—Since an atmosphere of carbon dioxide prevents the disintegration and volatilization of ammonium carbonate, tight covers are to be recommended for the conservation of solid and liquid manures. It has also proved useful to leave a small quantity of old, well fermented material in the pit when the manure is removed to the field. As soon as fresh material is added to the old manure a vigorous fermentation sets in, due to the inoculation taking place from the old to the new manure, and the desired atmosphere of carbon dioxide is soon restored.

If large quantities of whey, molasses, or of other waste products rich in soluble carbohydrates are available, they may be added to the manure; they are quickly transformed therein to lactic and other organic acids, which contribute to the fixation of ammonia. A similar and perhaps more efficient biological acid production in manure may possibly be effected by adding commercial sulfur to the manure. The sulfur is rapidly oxidized to sulfuric acid which is neutralized by the ammonia. Tests made at the Ohio Experiment Station gave very promising results. Manures kept for 250 days without and with sulfur showed the following losses:[1]

Percentage	Untreated	With Sulfur
Organic substances	32.5	18.0
Nitrogen	10.5	3.5

If these findings are confirmed, and if the cost of such treatment is not too high, this method would deserve careful consideration.

[1] J. W. Ames and T. E. Richmond, *Soil Science*, vol. 4, 1917, pp. 79–89.

CHAPTER XIV

BACTERIA AND RELATED MICROORGANISMS IN SOILS

Physical, chemical, and biological factors are active in the primary formation of soils from rocks, as well as in the secondary transformations continually taking place in all soils. Higher and lower plants and animals participate in these processes; soil bacteria and related microorganisms constitute a very important group among them. Like all living organisms they are influenced by the physical and chemical conditions prevailing in the soils, but they are also able to change these conditions to a greater or less extent. Just as the visible plant growth is dependent on the productivity of the soil, and causes at the same time either an increase or a decrease in fertility, so the microflora, too, is passively and actively closely connected with the special condition of a soil. Because of the numerous and variable differences in the structure and composition of the soils many physical and chemical problems are still to be solved, and in soil microbiology much more remains to be done, since such work has been under way only during forty or fifty years. Thus far a general survey has been made, and the more important subjects have been studied.[1]

1. GERM CONTENT OF SOILS

Bacteria are of greatest importance in all soils in regard to their number as well as to their activity. Fungi and protozoa are usually less frequent, but occasionally they, too, may become quite conspicuous. The same is true with the algae in soils. Like the protozoa they do not find, as a rule, enough moisture in field soils, for their requirements in this respect are generally higher than those of the bacteria, and especially of the lower fungi. In addition to the water content, the aeration and temperature of the soil, as well as its reaction and food supply, are the main factors regulating the "life" of a soil. Because these conditions are continually changing in every soil, it is quite obvious that the microflora of a certain soil is by no means constant.

[1] For detailed references see F. Löhnis, "Handbuch d. landwirtschaftl. Bakteriologie," Chap. V, and *Centralbl. f. Bakt.*, II. Abt., vol. 54, 1921, p. 285.

Season, rainfall, tillage, manuring, and cropping may cause far-reaching modifications. Investigations in which these important factors are not adequately considered are of very little value, and the germ content of a soil can not be accurately determined by one or a few simple bacteriological tests. Such studies should be continued for at least one year.

Frequency of Microorganisms.—Some general statements about the number of bacteria and of other microorganisms present in soils have been made on p. 60. There it was also pointed out that these high figures should not be overrated. The minute size of the bacteria and their uneven distribution in the soil must always be kept in mind. The total weight of the soil organisms, however, is quite remarkable and clearly indicates that far-reaching physical and chemical effects may be expected from such a mass of living matter.

Bacteria and related microorganisms are present in every soil from the tropics to the polar regions, and even desert sands and alkali soils which are almost devoid of higher plant growth, have their microflora. Of course, fertile soils are more thickly populated and give rise to a much more varied microflora, but even in such soils it is, as a rule, only the upper layer, relatively rich in humus, that offers a most suitable environment. One hundred to 1000 million of bacteria, fungi, algae, and protozoa have been found in fertile soil; more frequently 5 to 50 millions have been counted per g. soil, because on the plate cultures, which are used as the basis for such calculations, only part of the viable microorganisms will produce visible colonies. There is no possibility of inducing all bacteria present in a soil to grow on any one artificial substrate, and all results obtained are therefore merely of relative value. They have, however, very clearly shown that the frequency of microorganisms rapidly decreases in all subsoils, and that this decline is more pronounced in the heavy and humid soils, than in the light and arid ones, mainly because of the better aeration in the latter soils. Higher temperature and more moisture are favorable to a rapid multiplication of the bacteria in the soil; nevertheless, a reduction in number and activity is frequently noticeable during the warmer season. Although other factors may cause modifications of this rule, it is usually during spring and autumn that maximal growth and activity occur in the soils. The only exception is fallow soil, where the maxima have mostly been found during summer. Frozen soil often gives exceptionally large numbers of colonies on the plates, but this is merely due to the breaking up of the colonies present in the soil;[1] the situation here is similar to that found in centrifuged milk.

[1] A. F. Vass, Cornell Agr. Exp. Stat. *Memoir* 27, 1919.

Physiological Groups of Soil Organisms.—Among the organisms growing in plate cultures *non-sporulating rods* are usually most frequent; liquefying and non-liquefying Fluorescentes, B. aerogenes, Proteus, and different yellow rods are found quite regularly. *Micrococci* are less numerous, and also the *sporulating bacilli* are not as frequent as might be expected. *Actinomycetes* are always present; especially those producing a soluble dark brown pigment are very characteristic on soil plates (see Fig. 1, Plate III). Their number may reach 50 per cent of the total figure when the soils tested have recently received an application of barnyard manure or of straw. *Fungi* predominate in acid soils, especially if they are rich in humus; forest soils show, therefore, a vigorous mold growth. *Protozoa* and *algae* are rare in dry, but frequent in wet soils; irrigation causes a strong development of these organisms. In general it may be assumed that in field and garden soil for every 1000 bacteria, 10 to 100 fungous cells, and 1 to 10 protozoa and algae may be present.

The microorganisms growing upon the substrates commonly used for plate cultures in the laboratory represent, however, only a part of the microflora of the soil, and very important physiological groups of soil organisms, such as the nitrifying and nitrogen fixing bacteria do not grow at all, or only to a very limited extent under these conditions. Special tests must be made in order to secure information upon their distribution. From such tests it was found, for example, that a field soil from which 50 million bacteria per g. were grown on the gelatin plate, showed the following development of the more important groups per g. soil, if this was used for inoculating suitable solutions:[1]

75,000,000 in peptone solution	162,500 denitrifying bacteria
25,000,000 in urea solution	100,000 nitrifying bacteria
2,500,000 nitrogen fixing bacteria	

But even such detailed figures are not of great value. It is not the number, but the activity of the soil organisms which is of importance, and the latter depends on the former to a limited extent only. High or low efficiency of the individual cells determines the result more than does the number and the species.

Relation Between Germ Content and Productivity of Soils.—In exceptional cases the bacterial number obtained by plate cultures may display a certain parallelism with the productivity of a given soil. For example, number and productivity are low in distinctly acid soils, and both will rise as soon as lime, manure, or nitrate are applied; but in soils of normal reaction no fixed relations exist between bacterial

[1] W. Millard, *Centralbl. f. Bakt.*, II. Abt., vol. 31, 1911, p. 502.

counts and crop production. The indiscriminate counting of soil bacteria is indeed no better than it would be to enumerate all green plants growing on an acre of land, quite irrespective of the kind of plant whether weeds or cultivated plants; but even if only the latter were singled out, and it would be found, for instance, that there are two million wheat plants on a given area, such a result would not be of great interest to the agriculturist. Always it is the efficiency which really counts, that is, the crop production and the activities of the soil organisms. Naturally, parallelisms may occur between number and efficiency, but more frequently they are absent. For example, numbers and metabolic effects of those five important physiological groups mentioned above were determined in soil samples taken from the same plots in January and in July. The July data presented below are calculated in per cent of those obtained in January:[1]

	Ammonification		Nitrification	Denitrification	Nitrogen-fixation
	From Pepton	From Urea			
Numbers.....	125	100	30	100	3000
Effects.....	113	308	77	113	80

It is readily admitted that all such figures are but approximately correct, because no method is known that would furnish quite exact results; but from all what is known concerning the widely differing and variable efficiency of all microorganisms, it is practically self-evident that no fixed relations can be expected between the productivity of soils and the number of bacteria living therein.

If the metabolic effects produced by the soil bacteria are determined under different conditions much more valuable conclusions can be drawn concerning the productivity of the soils tested. It is to be kept in mind, however, that the activities of soil organisms are greatly influenced by the season; they are generally lowest during winter, high in spring and in autumn, and again lower in summer. In Fig. 55 a few annual curves are reproduced which show very clearly these variations and irregularities.[2] Frequently spring and autumn maxima have been observed, but weather, tillage, liming, manuring, and cropping may cause so many exceptions to this rule that it is quite indispensable

[1] F. Löhnis, *Centralbl. f. Bakt.*, II. Abt., vol. 14, 1905, p. 6.

[2] F. Löhnis, *Mitteilungen d. Landwirtschaftl. Instituts d. Univ. Leipzig*, Heft 7, 1905.

to extend such investigations through different seasons, and never to rely upon short-termed and isolated observations.

The productivity of a soil depends on its chemical, physical, and biological conditions. The first ones are relatively stable, the second ones rather variable, and the third ones very unstable. Most of the potential plant food in the soil is insoluble, and therefore not directly accessible to the plant roots; but at the same time it is protected

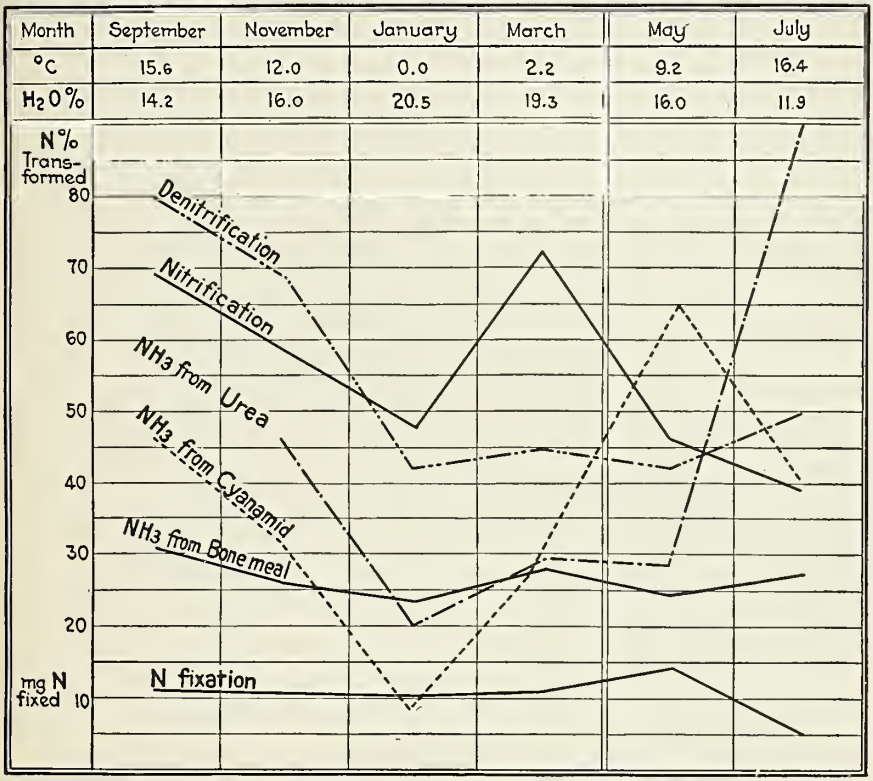


Fig. 55.—Annual curves of nitrogen transformations.

against being lost by leaching. Gradually and continually relatively small parts of this large stock of inert material are being made available, mostly by the direct and indirect action of soil organisms. The transformation of carbonaceous and nitrogenous substances is almost exclusively the work of bacteria and fungi, while the dissolution of minerals depends in the first place on the degree to which the soil solution is saturated with carbon dioxide, which again is largely produced by the minute inhabitants of the soil. Temperature, mois-

ture, tillage, and liming are of greatest influence upon the physical conditions of a soil, but the growth and incessant activity of the soil organisms are also not negligible in this respect. The difference in the productivity of surface soils and of subsoils, which is usually most pronounced in heavy soils under humid conditions, demonstrates very clearly the eminent importance of the "life" in the soil.

Soil Sickness.—Sometimes it happens that despite heavy applications of manure and fertilizers the productivity of a soil shows a marked decrease, especially as far as certain crops, such as clover, peas, lupines, flax, cucumbers, and grapes are concerned. It is customary to speak in such cases of clover sickness, etc., or more generally of a sickness or a fatigue of the soil. Frequently there are real causes of "sickness," that is, a multitude of disease producing organisms, fungi as well as insects, have accumulated in the sick soil, because of a too often repeated planting of the same crop. In other cases large quantities of fertilizers may have been applied, but not in proper relation to each other; too much lime, for instance, curtails the availability of potassium for certain plants, like lupines and flax, so much that they may become "lime sick." However, even if all chemical as well as physical conditions are well taken care of, as in greenhouses, and no damage is done by disease producing organisms, another fatigue of the soil may arise which is due to an excessive development of soil protozoa that prey upon the soil bacteria. As was said before, protozoa are favored by a high moisture content of the soil; in greenhouses and in irrigated fields the situation is most favorable for them. Bacteria are their main source of food, and reciprocal increases and decreases of protozoa, especially of amoebae, and of bacteria are very conspicuous under such conditions.[1] Figure 56 shows two such animals taken from a crude culture of Azotobacter, whose large globular cells have been in part ingested.

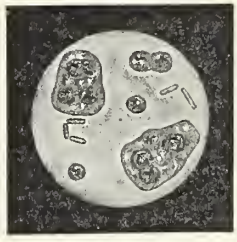


FIG. 56.—Protozoa in a crude culture of Azotobacter, living, ×1000.

However, not every reduction in number and activity of the useful soil bacteria can be explained by the predatory habit of soil protozoa. A heavy growth of cultivated plants may also cause such a reduction, either by the lowering of the moisture content of the soil, or by the production of a considerable amount of organic substances that act unfavorably upon many soil bacteria, as well as upon higher plants.

[1] D. W. CUTLER and L. M. CRUMP, *Annals of Applied Biology*, vol. 7, 1920, p. 11.

It is an old practical experience that many soils need a rest after a crop was taken, in order to regain their normal productivity, and it has also repeatedly been ascertained that the biological activities in the soil have the tendency to decline during the summer in cropped fields, while they do not decrease in fallow. That this is partly due to the presence of soluble organic substances, which can be removed by leaching, has been shown experimentally,[1] and very probably soil sickness is also caused to some extent by such accumulations which under ordinary conditions are decomposed by soil organisms before the next crop is planted.

Biological Soil Tests.—It is a comparatively simple matter to make plate cultures, to show the presence of microorganisms in a soil, or to add different substances to samples of soil, to keep them for a few weeks, and then to prove by chemical analysis what transformations have taken place in these samples as compared with others that had not been treated or had been sterilized before. However, it should never be overlooked that all experiments in the laboratory are made under conditions which are very different from those regulating the life of the soil organisms in the field. Therefore, in drawing any conclusions or basing any generalizations upon such results very great care should be exercised. It has been emphasized that because of the instability of bacterial activities all short-timed biological soil tests are of little value; furthermore it should always be kept in mind that because laboratory tests are invariably conducted under conditions differing from those in the greenhouse or in the field, they should be coordinated as far as possible with pot and plot experiments, whose results will help to prevent incorrect generalizations and will materially increase the value of such tests.

For preparing solid and liquid substrates for soil organisms an extract made by heating soil with an equal amount of water, has proved very useful. The chemical qualities of the particular soil to be tested are thus in part retained in the bacteriological test, and very frequently soil extract has been found to be a much better substrate than any artificial solution, because too little is known about the real composition of the soil solution, as well as about the most suitable growth conditions of the various groups of soil organisms. Meat substrates and blood serum are usually best for the cultivation of the bacteria living in man and animal; solutions prepared from pure chemicals dissolved in distilled water are usually much inferior. With soil organisms the situation is very similar.

[1] A. Lumière, *Compt. rend. Acad. Paris*, vol. 171, 1920, p. 868.

Occasionally the opinion has been expressed that the activities of soil organisms could be studied accurately only in the soil itself, and that any solution, made either from soil extract or from pure chemicals, be unsuitable. However, careful consideration of what is known at present about bacteria shows very clearly that almost every discovery since the time of Leeuwenhoek, including all information about ammonifying, nitrifying, denitrifying, nitrogen assimilating, and other organisms, has been derived from cultivating these organisms in properly prepared solutions.[1] In the soil itself the bacteria live in the solution circulating around and between the solid particles. Undoubtedly, tests made in soil are of value, too, but because of the complex and largely unknown composition of this substrate the results obtained leave much to be desired.[2] In all such tests the chemical and physical conditions in the soil samples are so different from those prevailing in the greenhouse or in the field that again no rash conclusions or generalizations would be permissible. If the soil is dried before being used, and excessive quantities of the substances to be tested are added to it, misleading results are almost inevitable.[3]

2. BACTERIAL ACTIVITIES IN SOIL

In the soil all organic residues are mineralized by the activities of bacteria and related microorganisms. Carbonaceous and nitrogenous compounds of almost endless variety are being decomposed, and many intermediary products are formed that at the present time are not well known. Especially concerning the dark colored soil constituents, collectively called humus, comparatively little has been ascertained. Much more complete data are available in regard to the metabolism of nitrogen; the relative scarcity of this element in agricultural soils, its high economic value, and its evasive nature have necessarily attracted the attention of all investigators. Since the nitrogen transformations are largely influenced by the quantity and quality of the carbonaceous substances present in the soil, the metabolism of the latter is also of great interest. These problems have been discussed in their general aspects in Chapter VII, 2-4; on the following pages

[1] M. W. Beijerinck, *Jaarboek d. Akad. Amsterdam*, 1913.

[2] F. Löhnis and H. H. Green, *Centralbl. f. Bakt.*, II. Abt., vol. 37, 1913, p. 534; vol. 40, 1914, p. 457.

[3] W. P. Kelley, *Jour. Agr. Research*, vol. 7, 1916, p. 417. Further details upon biological soil tests are given in the authors' laboratory manuals mentioned on pp. 11 and 12. In regard to experiments on soil protozoa see N. Kopeloff, H. C. Lint and D. A. Coleman, *Centralbl. f. Bakt.*, II. Abt., vol. 45, 1916, p. 230; and D. W. Cutler, *Jour. Agr. Science*, vol. 10, 1920, p. 135.

they will be considered in their relations to soil fertility. In the transformations of mineral substances, such as phosphates, potassium salts, etc., bacterial activities are, as a rule, of secondary importance, as has been explained in Chapter VII, 5.

Carbon Metabolism.—The quantities of organic substances which remain in the fields or are returned to them in the form of organic manures, are quite considerable. The dry weights of crop residues, that is, of stubble and roots, vary according to the intensity of cultivation usually between 800 and 3000 lbs. per acre. An average dressing with stable manure furnishes approximately 6000 to 8000 lbs. of organic substances, while in the form of green manures 2000 to 10,000 lbs. may be added, dependent on the growth of these plants. The total dry weight of carbonaceous substances returned annually to the soil, varies according to the type of farming, between 1000 and 6000 lbs. per acre; 3000 lbs. may be accepted as a moderate average for fields receiving organic manures regularly, though not abundantly.

Carbon dioxide and humus are the main products formed by bacterial action from all these organic residues. Organic acids, alcohols, methane, and hydrogen may appear as by-products, but the last-named gases are practically absent in well-aerated soils, while they are regularly present in swampy lands, wherein the organic acids are also more frequent. Strong aeration and high temperatures favor the formation of carbon dioxide, while under the opposite conditions large quantities of very resistant humus are produced. Both extremes are detrimental to the fertility of the soil, which is best maintained if a moderate amount of neutral humus is always retained and rebuilt in the soil, as is done in garden land and in highly productive fields.

Formation of Carbon Dioxide.—If annually 3000 lbs. of organic matter are returned per acre, and the humus content of the land is sufficiently high, so that these organic substances can be oxidized to carbon dioxide without depleting the soil, approximately 6000 lbs. of this gas will be produced, if 50 per cent is accepted as the average carbon content of the organic substances. The volume of these 6000 lbs. of carbon dioxide is approximately 1600 $m.^3$; if all this gas would be produced simultaneously and could be kept together, one acre of land would be covered by a uniform layer of carbon dioxide 16 inches deep. It is obvious that these large quantities of carbon dioxide must be of great influence upon the crop production. On the one side the green plants are supplied with carbon dioxide for their assimilation; on the other side the mineral constituents of the soil are attacked by the carbon dioxide dissolved in the soil solution, and thus inert plant food is made soluble. The roots of the cultivated plants also

produce carbon dioxide and participate in the dissolution of the minerals in the soil, but the quantities of carbon dioxide produced by the soil organisms are, as a rule, much larger, provided enough organic substances are present in the soil, or are regularly returned to it.[1]

After a heavy application of barnyard or green manure the production of carbon dioxide is sometimes so vigorous that the soil atmosphere temporarily loses all of its oxygen. Ten per cent of carbon dioxide have been found frequently, but the lowered oxygen tension is still sufficient for a normal nitrification and at the same time most favorable for the formation and conservation of humus in the soil. Since high soil temperatures and ample, though not excessive, soil moisture stimulate the respiration of the soil organisms, the resulting increase and decrease of carbon dioxide production may be used as a fairly reliable measurement of the bacterial activity of a soil. Quite exact results, however, are not obtained, because the removal of the soil samples from the field changes the physical conditions to a smaller or larger extent, dependent on the manner in which the samples are taken and handled. Small quantities of carbon dioxide may be produced by sterilized soil, too, but they are practically negligible.

Decomposition of Different Carbon Compounds.—Part of the organic substances, for instance, straw, corn stalks, stubble and roots, solid excrements, and peat litter are decomposed slowly; they produce more humus than carbon dioxide. Other substances, such as young green plants, are quickly disintegrated, and most of their carbon is oxidized to carbon dioxide; green manure plowed under during summer vanishes often entirely from the soil before the next crop is planted. The following figures show how much of the carbon content of various substances was oxidized within 3 weeks, if these were added in every case at the rate of 10 g. carbon to 500 g. of soil.[2]

	Per Cent		Per Cent
Red clover	59.69	Oak leaves	17.70
Glucose	42.14	Wheat straw	14.54
Starch	29.00	Cellulose	11.77

Because of the comparatively large quantities of carbon added to the soil in this experiment, the data obtained are merely of relative value. In the field much smaller amounts are applied, and the transformation is more rapid. For example, in laboratory experiments where 0.5 per cent of cellulose was mixed with soil, 70 to 100 per cent of it was

[1] E. B. Fred and A. R. C. Haas, *Jour. General Physiol.*, vol. 1, 1919, p. 631.

[2] J. Dvořák, *Zeitschr. f. d. landw. Vers. Wesen in Oesterreich*, vol. 15, 1912, p. 1097.

decomposed within one month.[1] Under field conditions usually not more than 0.1 per cent of the soil weight is added annually in the form of organic residues, but because all transformations proceed more slowly in the field than in the laboratory, these relatively small quantities suffice, as a rule, to maintain the carbon balance in the soil.

The decomposition of cellulose in field soils is usually done by aerobic bacteria and fungi. If sheets of paper are placed on top and 8 to 10 in. below the surface in soil whose water holding capacity is fully saturated, after 2 to 3 weeks the decomposition is very marked in the first, but hardly noticeable in the second case. Figure 57 illustrates this fact. Because a very large number of soil fungi are capable of dissolving cellulose, their growth is greatly enhanced if this substance is added to the soil. It has been observed, for instance, that

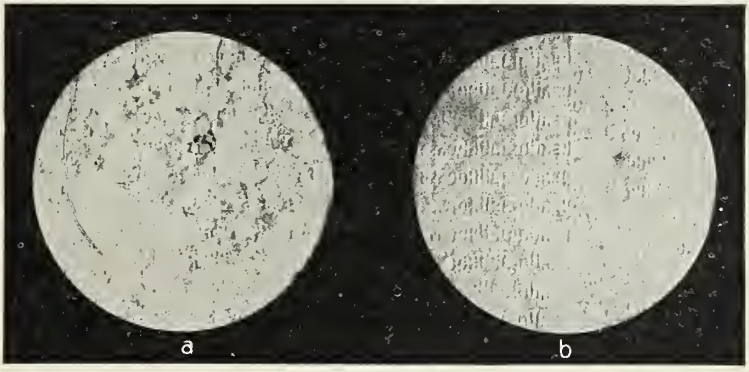


FIG. 57.—Cellulose decomposition in soil (*a*) at the surface, (*b*) 8 in. below the surface.

under such conditions their number rose from 100,000 to 200,000,000 per g. soil.[2]

Formation of Humus.—A larger or smaller part of the organic residues incorporated in the soil is transformed into humus. If no cultivation takes place, as in grassland and forests, the humus content shows a gradual and continuous increase, and if this process extends through long periods as in virgin soils, enormous quantities of humus are accumulated which constitute an extremely valuable store of potential plant food. Unfortunately, after such soils have been taken under cultivation their original fertility has frequently been wasted, and lack of humus is now often the main cause of the reduced productivity of such land.

[1] C. MÜTTERLEIN, *Studien über die Zersetzung der Zellulose*, Diss. Leipzig, 1913, p. 96.
[2] F. M. SCALES, *Botan. Gazette*, vol. 60, 1915, p. 149.

Beeause praetieally all nitrogenous as well as non-nitrogenous organic substances ean be changed to humus, the chemical eomposition of the latter varies in all soils. Carbohydrates, especially eellulose, are usually completely deeomposed if fully aerobic or strictly anaerobic eonditions favor the aetion of baeteria. Fungi and actinomycetes, on the other hand, are more inclined to produee dark humus-like bodies when growing on cellulose (see Fig. 2, Plate VIII).

Mueh of the finely divided, rich humus of field and garden soil is made up of the exerements of worms and insects which constitute an important part of the soil fauna. They all feed on the organic residues upon and within the soil, and this transformation of plant substance into animal exerements plays by no means a subordinate rôle. Counts made at the Rothamsted Experimental Station showed that per acre 4 to 10 millions of insects, worms, and myriapods were present in field soil; manuring eaused a marked increase in their number.[1]

Decomposition of Humus.—If no organie manures are used, and only the crop residues are left on the field, sooner or later the soil will be robbed of most of its humus and of its natural fertility. Intensive clean cultivation, as practieed in eorn fields and orchards, together with high summer temperatures aceelerate this process unavoidably; accordingly, vast stretehes of American soil have lost much or nearly all of their original humus content. Under average eonditions 4 to 5 per cent of the humus are deeomposed annually; that is, in a soil with 2 per eent humus approximately 3000 to 4000 lbs. per aere. From what was said above coneerning the carbon metabolism it is quite evident that these losses of humus neeessitate the intelligent use of barnyard and green manures.

The decomposition of the earbon in humus proeeeds generally more rapidly than that of its nitrogen; old humus is therefore, as a rule, richer in nitrogen than is that of more recent origin. Under average conditions 1 to 2 per eent are nitrified annually; in soil eontaining 0.1 per cent of nitrogen this is equivalent to 40 to 80 lbs. of nitrogen per acre. In prairie land the nitrogen content is frequently considerably higher, and often much more humus nitrogen is nitrified than can be used by the crops, whieh results in great losses by leaehing. Humus of different origin may behave very differently in regard to the nitrifieation of its nitrogen.[2] Stable manure, green manure, peat, and straw were kept in elean sand for 4½ months; the humus formed

[1] Rothamsted Exp. Stat. *Report* 1918–20, p. 20.

[2] F. Löhnis and H. H. Green, *Centralbl. f. Bakt.*, II. Abt., vol. 40, 1914, p. 56.

was then extracted and mixed with soil. After 5 weeks the nitrate nitrogen was determined and calculated in per cent of the total nitrogen:

Percentage	Humus from			
	Stable Manure	Green Manure	Peat	Straw
Total nitrogen.....	3.53– 3.67	4.16	2.29	1.34
Nitrate nitrogen.....	11.2 –16.3	14-18	2.9–3.8	0

The great resistance of peat nitrogen is very marked. The straw humus was not nitrified at all, and it even caused a reduction in the nitrate content of the soil, although not to the same extent as is characteristic of fresh straw.

Soil Acidity.—Unless neutralized by lime, most of the humus is of distinctly acid reaction, and because many of the processes connected with the transformation and mineralization of organic residues lead to the formation of acids, there is a natural tendency at least in humid soils not naturally rich in lime to become more or less acid. Absorptive processes and mineral acids contribute to the soil acidity, but wherever humic acids are present in considerable quantities they always play a prominent rôle. If they are not neutralized, the humus decomposition remains very low, because of the inactivity of the majority of soil organisms. As soon as the reaction is adjusted by liming, a vigorous bacterial activity sets in even in peat soils, and excessive liming may sometimes become detrimental because of too rapid decomposition of the humus and great losses in nitrogen.

Tilth and Ripening of the Soil.—Formation and decomposition of humus play a very important rôle in securing the desired tilth of the soil. This particular condition which is essential for obtaining regularly large and healthy crops, can not be established merely by careful tillage of the soil. Good tillage is very necessary, but the best possible physical condition of the soil is not secured by mechanical treatment alone. If the soil is covered with a thick layer of organic residues, as in virgin woodland and in mulched orchards, it will often exhibit a physical structure superior to that of cultivated land. Such soil is not loose, but of a peculiar, crumbly and elastic, mellow texture, which is due to colloid reactions as well as to the direct action of all the worms and insects that participate in the formation and transformation of the humus. The moisture content of covered soils is

higher and more evenly distributed than in cultivated land, and the chemical and biochemical processes are therefore, as a rule, greatly stimulated. Noxious organic substances are rapidly decomposed, inert plant food is made soluble, and perfect tilth is ultimately established which comprises the best possible physical, chemical, and biological conditions of the soil, and thereby furnishes a reliable basis for securing large and healthy crops.

It always takes time before this aim is reached. If the climatic conditions are favorable, if tillage and manuring are done efficiently, if the soil reaction is right and the humus content sufficiently high, the desired tilth can be attained within 2 to 3 months. If the climatic conditions are unfavorable, if the physical structure of the soil is bad and its biological activity low, a longer time is needed, and in extreme cases, when the crop production becomes entirely unsatisfactory, the field must be kept and treated as fallow for a full year.

A soil which is not in good tilth is properly termed "raw"; if it is planted the crop will be comparatively low. Soil in good tilth on the other hand may be called "ripe," because it is ready to produce a good crop, and the slow processes that lead to this stage constitute in their entirety a real "ripening" of the soil. In the European agricultural literature the problem of how to obtain a perfect tilth of the soil has been discussed extensively, and the term tilth (in German "Gare") has frequently been interpreted as meaning "fermentation." Because carbon dioxide is produced and the structure of the soil becomes soft and elastic, the tilth of the soil has been compared with the leavening of bread; but, the two processes are, in fact, rather different. It is not alone a fermentation, but a general mellowing or ripening of the soil that takes place.

Nitrogen Metabolism.—Comparatively little plant food enters the soil in directly available form; nitrate, acid phosphate, and potassium salts are fertilizers which are immediately accessible to the plant roots. Nearly all other fertilizers and manures, as well as all crop residues, must first be transformed by bacterial action. When incorporated in the soil, they represent sources of food and energy for the minute inhabitants of the soil, and only the metabolic products of the microorganisms are taken up by the cultivated plants. This indirectly fertilizing effect of all organic substances, and especially of all organic nitrogenous compounds is the reason why the nitrogen given in this form never produces such prompt and uniform results as are obtained by the application of nitrate. The more complex the nitrogenous compounds are, as in barnyard manure, green manure, horn meal, and bone meal, the more stages of the nitrogen cycle are to be passed before

nitrate is produeed; and the more opportunities are offered to the soil organisms to use the nitrogen for their own purposes, the less nitrogen becomes available for final nitrification. It is of great practical importanee to find out under what eonditions these transformations proceed in the soil in sueh a manner that the most satisfactory fertilizing effect will be realized, but not very much is known about this subjeet, and the major part of this work remains to be done.

Formation of Ammonia from Farm Manures.—It was pointed out in the preeeding chapter that the mineralization of the nitrogen in barnyard manure is almost completely eheeked if the material is plowed under before the carbonaeeous substanees are partly decomposed, and it was also emphasized that a prompt fertilizing effeet is to be expeeted only from the liquids. These important faets are further illustrated by the following results obtained in nitrification tests made with manure of different age.[1] The materials were mixed with soil and the inerease in mineral nitrogen (ammonia and nitrate) was determined after 6 weeks and calculated in per eent of the total nitrogen added in the manure:

Percentage of Nitrogen		Manure Used			
		Fresh	1 Wk. Old	2 Wks. Old	4 Wks. Old
Mineralized from	straw and feces.....	2	6	21	31
	straw, feces, and urine	7	31	58	75

Because in the field the conditions for baeterial action are much less favorable than in the small soil samples used in the laboratory, these data are again of merely relative value. Under field conditions rarely more than 10 to 20 per eent of the nitrogen in straw and feees are mineralized within the first year, whereas 30 to 50 per eent may beeome available if liquids and solids had been mixed, and 60 to 80 per eent if the liquid manure was applied separately. Usually the transformation of the urine nitrogen to ammonia is eomplete before the liquid manure is brought out to the field. If the ammonia has not been fixed by sulfuric acid or by formaldehyde, as was reeommended on p. 235, large quantities of it may be lost when the liquid manure is spread and left on the surface.

With green manures similar differences in the mineralization of nitrogen may be observed. Variations of the fertilizing effect between

[1] F. Löhnis and J. H. Smith, *Fühling's landw. Zeitg.*, vol. 63, 1914, p. 153.

15 and 85 per cent are not infrequent. As a rule, the nitrogen of young plants is more easily mineralized than that of older plants. because more of it is present in the form of amino nitrogen, and the nitrogen-carbon ratio is more favorable to the ammonifying bacteria. If old material rich in carbonaceous substances is used, a partial rotting is as advisable as it is with stable manure. If circumstances permit, it is better to leave such green manure over winter on top of the soil than to plow it under in fall. In China and in India special rotting processes have long been practiced in connection with green manuring. Neither barnyard nor green manure should be buried deep into the soil, because there the mineralization would be very incomplete. On the other hand, the decomposition will usually be too rapid, if young green plants are turned under during the hot season.

Formation of Ammonia from Organic Fertilizers.—Commercial organic fertilizers, such as dried blood, fish guano, horn meal, rape cake, and cyanamid, are also known to exert rather variable effects, because their nitrogen again must first be mineralized by soil organisms. If more laboratory tests would be made concerning the ammonification and nitrification of these fertilizers in different types of soil and under different climatic conditions, and if such tests would be connected with field experiments, the real value of the various organic fertilizers would be undoubtedly much better known than it is at present. The curves reproduced on p. 241, showed, for example, that in May, that is, at the time when most of the nitrogen was made available, in laboratory tests 25 per cent of the bone meal nitrogen and 65 per cent of the cyanamid nitrogen were ammonified. The percentage of fertilizer nitrogen taken up by the potato crop grown on this land proved to be 25.3 and 61 per cent respectively. Cyanamid and urea are undoubtedly the most valuable among the commercial organic nitrogen fertilizers. Cyanamid itself is poisonous to green plants and should, therefore, be applied several weeks before planting. Soil colloids effect a rapid transformation of cyanamid into urea, and the urea is changed to ammonium carbonate by bacterial action. Various soil fungi are capable of attacking cyanamid directly, but the rate of ammonification is rather low. Because of the much more rapid action of the soil colloids and the fairly complete ammonification which was regularly observed in laboratory tests, the first-named process is undoubtedly of greater importance. As a rule, non-sporulating bacteria are most frequent in ammonification tests, but sporulating bacilli, though less numerous, are often much more efficient. Ammonifying fungi dominate in acid soils; in neutral soils they are less conspicuous, though not absent.

Nitrification.—Many experiments have demonstrated that all green plants may assimilate ammonia nitrogen, and certain plants, such as rice, grow even better with ammonia than with nitrate; but the majority of cultivated plants prefer the nitrate nitrogen. Prompt nitrification of ammonia nitrogen is generally favorable, because ammonium carbonate may exert a caustic effect upon the plant roots, resulting in the "burning" of the plants, which is sometimes observed when liquid manure is brought in contact with growing crops. Such damage would be prevented only in acid soils and in those of very high absorptive power; in all other soils nitrification is distinctly useful.

Although the nitrifying bacteria prefer a slightly alkaline reaction, the average hydrogen-ion concentration in soils, which is usually somewhat below pH = 7, is still sufficient, and nitrification has been observed even in distinctly acid peat and forest soils. In acid soils the transformation is likely to proceed in a somewhat abnormal manner insofar as unusually large quantities of nitrite may accumulate, which may prove injurious to the cultivated plants. In normal soils hardly any nitrite can be found; both groups of nitrifying bacteria cooperate closely, and the ammonia nitrogen is oxidized to nitrate without any considerable loss. Two French agricultural chemists, Th. Schlösing, sr. and jr., have made interesting experiments upon this problem, which furnish a clear picture of the influence exerted upon nitrification by the composition and moisture content of the soil.[1] Sand and clay were mixed in different proportions, and so much water was added that in all cases it was equal to 9.5 per cent of the soil weight. Within a few months the following percentages of ammonia nitrogen were nitrified:

	Per Cent	Per Cent	Per Cent	Per Cent	Per Cent	Per Cent
The mixtures contained clay	0	10	15	20	25	30
The mixtures contained sand	100	90	85	80	75	70
Nitrogen nitrified	63	66	94	100	21	2.7

In the mixtures of high sand content probably part of the ammonia was lost by evaporation. That the low nitrification in the mixtures relatively rich in clay, on the other hand, was solely due to insufficient moisture, was proved by the following experiments. Somewhat larger amounts of water were added to the mixture containing 30 per cent

[1] *Compt. rend. Acad. Paris*, vol. 109, 1889, p. 423; vol. 125, 1897, p. 826.

clay and 70 per cent sand, and now the nitrification proceeded in the following manner:

	Per Cent	Per Cent	Per Cent	Per Cent
Moisture content	10.6	11.5	13.2	14.0
Nitrogen nitrified	80	100	100	100

Undoubtedly, the increase in nitrification which is frequently observed as a result of soil tillage, is much less due to an increase in aeration, than to an increase in the water-holding capacity and in the moisture content of the soil.

If the conditions are very favorable for nitrification, as is common in irrigated soils in arid regions, this process may assume unusual dimensions. Strong evaporation of the soil moisture may cause accumulations of nitrates in the surface soil, so-called niter spots, which sooner or later prove deleterious to the crop. Most of the nitrate formed under such conditions is derived from the decomposition of humus; one half or more of the total nitrogen content of these soils is sometimes made up of nitrates. Heavy mulching with organic substances is to be recommended for regulating the physical as well as the biological conditions of such abnormal soils.

Assimilation of Ammonia and Nitrate Nitrogen.—It very rarely happens that all the nitrogen given to the soil in the form of fertilizer is taken up by the following crop. As a rule, it must be accepted as a satisfactory result if approximately 60 to 80 per cent of nitrate nitrogen, 50 to 75 per cent of the nitrogen of ammonium sulfate, cyanamid, and liquid manure, 40 to 60 per cent of the nitrogen of green manures, and 20 to 40 per cent of that in barnyard manure are returned in the first year's crop. Part of the nitrogen in the form of nitrate is lost by leaching, but the pronounced inferiority of the organic manures is mainly due to their high content of carbonaceous substances which enable the microorganisms of the soil to assimilate the manure nitrogen as well as that present in mineralized form in the soil, and in this way to rebuild complex organic compounds.

If large quantities of straw are added to the soil, nitrate and ammonia are rapidly assimilated by bacteria and fungi. A new crop planted soon after will show all indications of nitrogen starvation. Under average conditions these processes are not of very great importance; about 3 to 5 per cent of nitrate nitrogen, and 10 to 25 per cent of ammonia nitrogen may be temporarily side-tracked in this

manner. Sooner or later the assimilated nitrogen will again be mineralized.

Losses of Nitrogen.—The bad effects frequently caused by straw and fresh stable manure have been repeatedly explained by the assumption that they are due to the detrimental activities of the denitrifying bacteria. It has been overlooked that an intensive denitrification can take place solely under strictly anaerobic conditions. It is true that the rapid formation of carbon dioxide from the straw and the saturation of the soil by heavy rains may temporarily establish anaerobic conditions in an otherwise well aerated soil. Under such exceptional conditions losses of nitrogen will occur, and occasionally this possibility may find practical application as a means of removing the excess of nitrate from land showing niter spots. Under normal conditions, however, the denitrification will not cause serious losses in the field. When the soil becomes water-logged as a result of heavy rains, the losses by leaching are probably always much larger than those due to denitrification.

Considerable quantities of ammonia nitrogen may be lost by evaporation in light calcareous soils, but this possibility may also become of importance in soils of higher absorptive power, especially in the application of liquid manure and of cyanamid. These substances should at the time of application be thoroughly mixed with or incorporated in the soil.

Fixation of Nitrogen by Nodule Bacteria.—Enough elementary nitrogen from the air is continually assimilated by bacterial action in virgin as well as in properly cultivated land that occasional losses of nitrogen are easily overcome, and the fertility of the soil is maintained or increased. The fixation of nitrogen by the bacteria in the root nodules of leguminous and some other plants is undoubtedly of greatest importance, as was discussed on p. 116. Figures 58 and 59 show a few characteristic types of root nodules. From the appearance of the nodules certain conclusions can be drawn concerning the distribution of the bacteria in the soil and their efficiency. Young roots only are invaded by the bacteria, and the more numerous and efficient these organisms are, the more numerous and better developed are the nodules in the oldest parts of the roots.[1]

The bacterial growth within the nodules is visible in Fig. 60. From the vascular bundle in the center of the root a sidebranch is seen to

[1] The normal root nodules are sometimes replaced by similar formations caused by *Bact. tumefaciens*, the organism of crown gall, which is purely parasitic. See U. S. Dept. of Agr., *Bureau of Plant Industry Circular* 76.

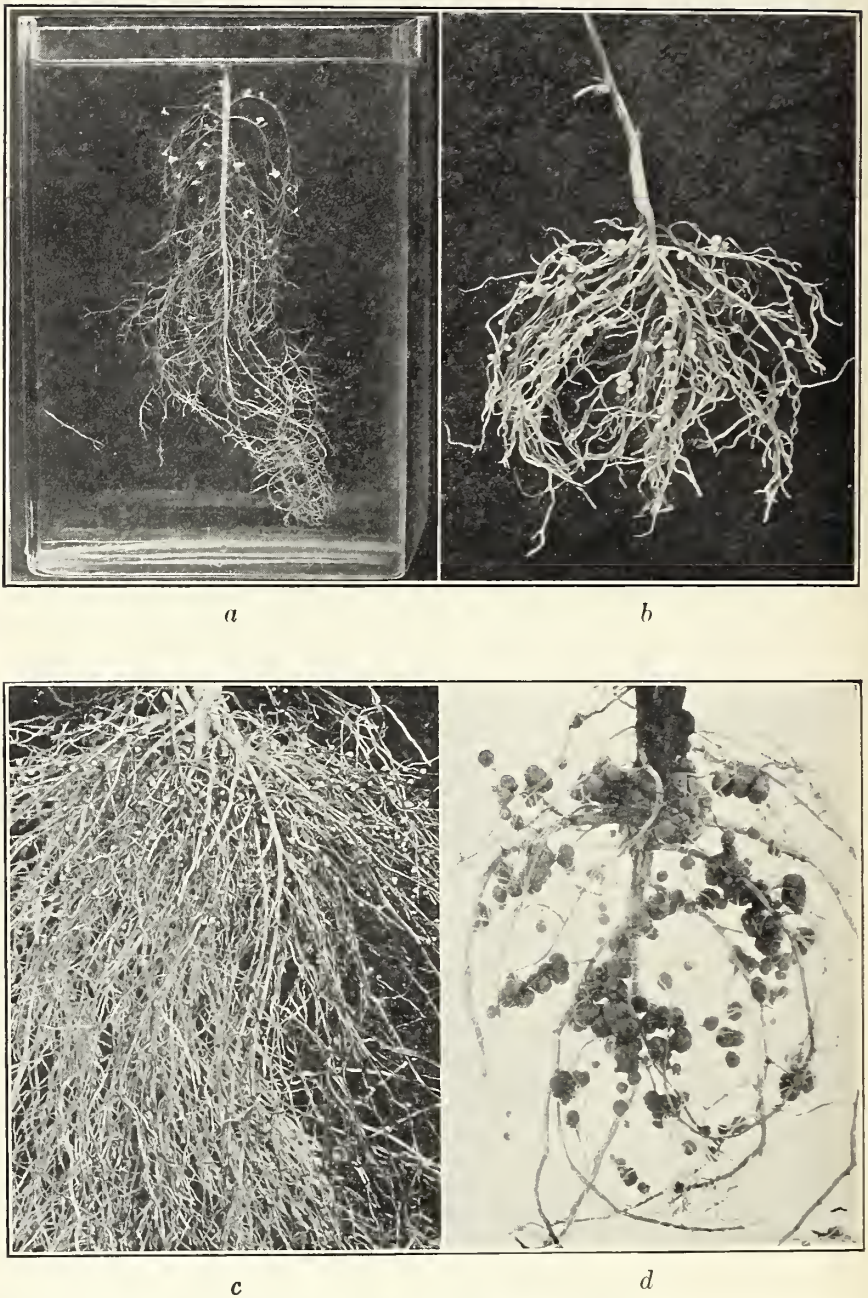


Fig. 58.—Roots with nodules of (*a*) young alfalfa, (*b*) young pea, (*c*) mature red clover, (*d*) mature soy bean ($\frac{1}{2}$ nat. size).

enter the nodule, where it spreads around the bacterial agglomeration. The carbohydrates needed by the bacteria are brought to them by this

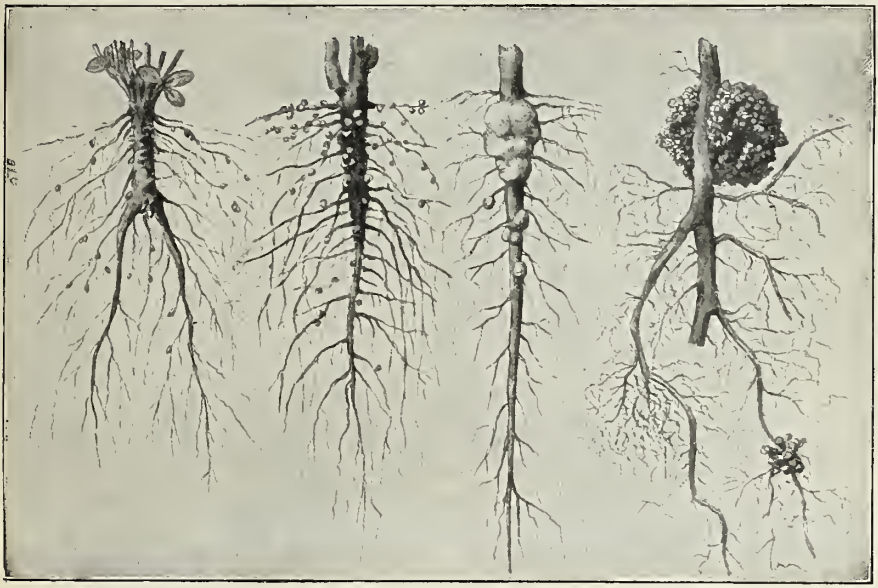


FIG. 59.—Roots with nodules of (*a*) red clover, (*b*) broad bean, (*c*) lupine, and (*d*) alder ($\frac{1}{3}$ nat. size).

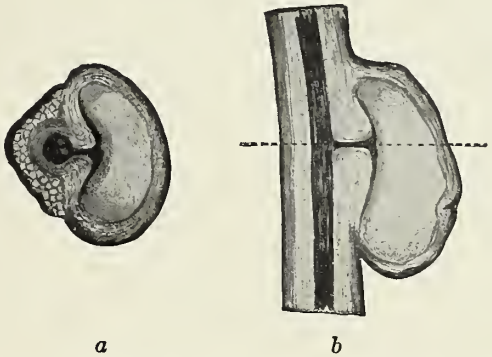


FIG. 60.—Sectional views of a root nodule, cut (*a*) lengthwise and (*b*) crosswise, ×20 The broken line indicates where the second cut was made.

channel, and the nitrogenous metabolic products are transferred to the host plant, as far as available.

The chemistry of the nitrogen fixation and its products is not yet

fully known; it is probably all protein that goes from the bacteria to the host. The nodules themselves retain a considerable part of the assimilated nitrogen, and this explains in part the beneficial effect which is often realized when leguminous plants are used as forage, and stubble and roots only are left on the ground. If the whole plants are turned under as green manure it may happen that their carbohydrates will check the mineralization of the nitrogen, and the fertilizing effect will perhaps be lower than in the other case. There are also economic reasons which make it advisable to use the legumes as feed, whenever possible.

Increase of Soil Nitrogen by Leguminous Crops.—A good crop of legumes contains approximately 100 to 200 lbs. of nitrogen per acre, but this amount does not represent the quantity assimilated from the air. If a soil is rich in nitrate, much of it is used by the leguminous plants and by the bacteria, and the nitrogen fixation remains relatively low; but even a poor soil furnishes some nitrate nitrogen, although the nitrogen fixation is very important in this case and very beneficial for the fertility of the soil. It has been well known for many centuries that poor and worn-out soils can be enriched most successfully and most economically by an intelligent use of leguminous crops, and persistent use should be made of these very valuable abilities of the nodule bacteria. Of course, the assimilated nitrogen is not clear gain, although it is often asserted that it may be secured without cost. The actual costs vary according to numerous calculations between 5 and 20c. per pound of nitrogen, and because the fertilizing effect of organic nitrogen is always below that of ammonium and nitrate nitrogen, proper allowance must be made for these differences, too.

To secure the highest possible benefit from a leguminous crop, it is necessary that the soil contains large quantities of phosphate, of potassium, and of lime if this is needed by the legume planted. Only healthy strong plants produce carbohydrates in abundance, and the nitrogen fixing bacteria are most active in their root nodules. Weak sickly plants show very few or no nodules, which fact demonstrates clearly that the relations between nodule bacteria and leguminous plants are truly symbiotic. If the bacteria acted as parasites, as is sometimes asserted, the most numerous nodules should be expected on the roots of sick plants, which is contrary to the facts observed. Soluble nitrogenous compounds should be absent in the soil, as far as possible; therefore, it is a good practice to use legumes as catch crops after cereals, which have depleted the soil of its nitrate.

Types of Nodule Bacteria.—If a legume is planted in a field where neither this nor related plant species have grown before, it may happen

that no root nodules are formed. Generally, nodule formation and nitrogen fixation are most satisfactory if the soil contains, or is supplied with the particular type of bacteria adapted to the special kind of legume. Clover grows best with clover bacteria, peas with pea bacteria, etc. Cross inoculations from one to another legume are rare with some, though frequent with other types of nodule bacteria. It is an open question whether or not the different types of nodule bacteria should be classified as separate species. The known differences in their general character are, as a whole, so inconspicuous that it seems preferable to group the strains isolated from different plants as more or less stable modifications or types of one species, which was described by Beijerinck as B. radicicola. This holds true especially for all strains that cause root nodules on the legumes which for centuries have been cultivated in Europe. They all have peritrichous flagella, and their cultural behavior is so much alike that they can not be differentiated, except by inoculation and agglutination tests. Somewhat greater differences become noticeable if, for instance, soy bean bacteria are compared with pea bacteria, but here again all cultures isolated from cultivated legumes of Asiatic origin behave alike. They have single, rarely several, polar flagella, and show uniform cultural features which are not very different from those of the first-named group.[1] It may be that these two groups really represent different species, but so long as their full life history is not known, no final decision can be made. Branched forms are generally more frequent in the peritrichous group (Figs. 2 and 7, Plate II), while such granulated cells as are shown in Fig. 61 are common in both groups.

FIG. 61.—Bacteria from alfalfa nodules stained, ×1200.

According to their ability to invade one or different legumes the nodule bacteria are to be grouped as follows:

A. Peritrichous type, found in cultivated legumes of European origin:

1. Red clover (Trifolium pratense), Alsike or Swedish clover (Trifolium hybridum), Crimson clover (Trifolium incarnatum), White clover (Trifolium repens), Cow clover (Trifolium medium), Berseem or Egyptian clover (Trifolium alexandrinum).

[1] F. LÖHNIS and R. HANSEN, *Jour. Agr. Research*, vol. 20, 1921, p. 543; I. SHUNK, *Jour. Bacteriol.*, vol. 6, 1921, p. 239.

2. Alfalfa (Medicago sativa and falcata), Bur clover (Medicago hispida), Black medick or yellow trefoil (Medicago lupulina), White sweet clover (Melilotus alba), Yellow sweet clover (Melilotus officinalis), Fenugreek (Trigonella fœnum-græcum).
3. Garden and field pea (Pisum sativum), Hairy vetch (Vicia villosa), Spring vetch (Vicia sativa), Broad bean (Vicia Faba), Lentil (Lens esculenta), Sweet pea (Lathyrus odoratus).
4. Garden bean (Phaseolus vulgaris and angustifolius), Scarlet runner bean (Phaseolus multiflorus).
5. Lupines (Lupinus albus, angustifolius, luteus, and perennis), Serradella (Ornithopus sativus).

B. Monotrichous type, found in cultivated legumes of Asiatic origin:
1. Soy bean (Soja max).
2. Cowpea (Vigna sinensis), Peanut (Arachis hypogæa), Japan clover (Lespedeza striata), Velvet bean (Stizolobium deeringianum), Beggarweed (Desmodium tortuosum), Kudzu vine (Pueraria thunbergiana), Lima bean (Phaseolus lunatus).

This grouping shows clearly that certain types of nodule bacteria are able to produce root nodules on very different plants, while other strains display a remarkably exclusive behavior. Occasionally, however, unusual cross inoculations have been recorded, for example between Medicago and Lupinus, Phaseolus vulgaris and Vigna sinensis, or Soja and Vigna, which are of great scientific though of no practical interest. The very sharp adaptations of the different types of nodule bacteria to their host plants are probably partly due to their preference for the special degree of acidity that is characteristic of the sap of these plants. The minimum hydrogen-ion concentration at which the bacteria still grew showed, for instance, the following interesting differences in the pH values:[1]

Medicago and Melilotus bacteria	5.0	Phaseolus bacteria	4.3
Pisum and Vicia bacteria	4.8	Soja bacteria	3.4
Trifolium bacteria	4.3	Lupinus bacteria	3.2

Certain facts seem to indicate that in the soil itself more or less neutral strains of nodule bacteria may occur, which will gradually adapt themselves if a new kind of legume that at first remains without nodules is planted continuously for several years. For all practical purposes, however, inoculation with adapted bacteria is strongly to be recommended.

Asymbiotic Nitrogen Fixation.—The fixation of nitrogen by Azotobacter and other soil organisms, which have been discussed in Chapter VII, 4, can never enrich the soil to such an extent as is possible by the growth of leguminous plants. Sometimes a symbiosis between

[1] E. B. Fred and A. Davenport, *Jour. Agr. Research*, vol. 14, 1918, p. 317.

bacteria and algae may improve the supply of carbonaceous material, but usually there is a decided lack of such substances in the soil, and the nitrogen fixing bacteria are therefore not in a position to display their full abilities. Approximately 100 parts of suitable carbonaceous material are necessary to furnish the energy for the fixation of one part of nitrogen. Under average conditions 3000 to 4000 lbs. of organic matter are annually available per acre. How much of this quantity is used by the nitrogen fixing bacteria, is not known; but it is evident that under unfavorable conditions perhaps 10, and under favorable conditions not more than 40 lbs. of nitrogen per acre and year can be expected from this source, that is, as was said before, $^1/_{10}$ to $^1/_5$ of what may be expected from the symbiotic nitrogen fixation in the legumes.

The results of bacteriological and chemical laboratory tests, as well as of long continued field experiments conducted in various parts of Europe, agree well with these calculations. A plus of 20 to 40 lbs. per acre and year has been regularly observed, when all data were carefully collected for drawing an accurate nitrogen balance. Much higher results have been recorded when the nitrogen content in uncropped land was determined after long intervals, as was done in Rothamsted in 1879, 1888, and in 1912. This soil showed an increase in nitrogen content from 0.205 to 0.235, and to 0.338 per cent.[1] Undoubtedly, wild growing legumes contribute to the total effect in such cases, and the carbon supply is also much larger than in cropped fields. In the tropics higher temperature is another factor which may stimulate the asymbiotic nitrogen fixation in the soil.

Conditions of Nitrogen Fixation in Soil.—Since the carbon metabolism in the soil affects the nitrogen fixation probably more than does any other influence, it becomes again very evident that the conservation and regeneration of the humus content of the soil requires most careful consideration. Many experiments have shown that under favorable circumstances, especially when the temperature was not too low, and sufficient time was allowed for the various transformations, the application of sugar or of other easily available carbonaceous substances stimulates nitrogen fixation very much. Two of such tests are reproduced in Fig. 62. Other promising experiments have been made with molasses, with peat previously treated with hydrochloric acid, and with other cheap carbonaceous materials; but no practical application of these results has thus far become possible, and not too much should be expected for the future.

[1] E. J. Russell, "Soil Conditions and Plant Growth."

Because a slightly alkaline reaction is most favorable for the growth of the nitrogen fixing soil bacteria, regular liming of all soils which show a tendency to become acid is much to be recommended. The application of basic slag or of acid phosphate also stimulates growth and activity of Azotobacter very distinctly; but no less important than the chemical condition of the soil is its physical structure. A soil in

FIG. 62.—Buckwheat and oats fertilized with sugar, after A. Koch. Pots 101 and 166 were not treated, 117 and 162 received sugar.

perfect tilth offers the most suitable environment to the nitrogen fixing as well as to all other useful soil organisms.

3. MEANS OF REGULATING THE ACTIVITY OF MICROORGANISMS IN THE SOIL

The bacterial activities can be regulated in the soil as everywhere else by direct and by indirect methods. Direct methods, that is, soil disinfection and inoculation, are applicable in exceptional cases only. The normal way of regulating the bacterial activity in the soil consists in the intelligent use of all those indirect methods which serve to maintain and to increase the productivity of the soils. Tillage, irrigation, liming, manuring, cropping, etc. are all of great influence upon

the environmental conditions under which the soil organisms live and work. The better these relations are understood, the better results will be secured in regulating the "life" of the soil, provided season and weather are favorable for the bacterial activities in the soil, as well as for the development of the cultivated plants.

Influence of Tillage.—Soil aeration, moisture content, soil temperature, and the distribution of the microorganisms and their food supply are more or less changed by every kind of mechanical soil treatment. Harmful anaerobic processes, such as denitrification, can not do great damage in a well tilled, that is, a well aerated soil. On the other hand, nitrification, the formation of carbon dioxide, and other aerobic transformations are often greatly stimulated by plowing and cultivating; but it is less the increased aeration than the change in other conditions that is of benefit to the active organisms. It was pointed out before that the desired aerobic processes are influenced very little when the oxygen tension is lowered to about one half of normal, and in regard to the formation and transformation of humus such semi-anaerobic conditions have proved most favorable.

The stimulating effect of plowing and cultivating is mostly due to the increase in the water-holding capacity of the soil and to the better distribution of the bacteria and of the organic residues in the soil. If the mechanical treatment is as thorough as it is in the filling of pots or in the preparation of soil samples for laboratory tests, a marked increase in germ content and in bacterial activities can always be noted (see p. 53). In the field, excessive cultivation or plowing at the improper time may also greatly stimulate the processes in the soil. The rapid loss of humus as a result of clean cultivation is well known. Nitrogen losses will be very large if the natural tendency of nitrification to reach a maximum in autumn is stimulated by thorough mechanical treatment of the soil at that time, and the nitrate formed is then washed away during a relatively warm, rainy winter.

If a soil is rich in humus and full of life, deep plowing and cultivating is to be recommended. In the opposite case the active surface soil should never be buried below an inert subsoil. Regular application of organic manures, subsoiling, and gradually deepening the fertile surface layer will greatly increase the productivity of such a soil. If the soil is loose and the climatic conditions favor the evaporation of water, this should be checked as much as possible by packing of the subsurface and by frequent harrowing, hoeing, shallow cultivation or mulching of the surface soil.

Influence of Irrigation and of Drainage.—The normal bacterial processes go on most satisfactorily in a soil whose moisture content

is equal to 60 to 80 per cent of the total water-holding capacity. Naturally, this optimum will not persist regularly, but because of the seasonal variations in bacterial activities it is very important that in spring and in autumn sufficient, though not too much, moisture should be present in the soil. If other means of regulating the water content prove insufficient, irrigation and drainage must be relied upon, according to circumstances. In addition to changing the moisture of the soil, both methods exert great influence upon its temperature, and in a lesser degree upon its aeration. As a rule, a well drained soil is a very active soil, provided excessive drainage has been avoided. A temporary drought is not very detrimental to the soil organisms; they become dormant, but are not killed. Frequently an increase in bacterial activities occurs after the soil has been thoroughly dried and remoistened; in irrigated land this effect is sometimes very marked. The improvement is partly due to a reduction in the number of soil protozoa, which are favored by excessive moisture and may do great harm to the bacterial flora in wet soils. Better aeration, increase in soil temperature, changes in the soil solution and in the condition of the soil colloids contribute to the favorable effect.

Influence of the Application of Organic Manures.—The regular application of organic manures exerts in all soils which are not very rich in humus the most pronounced influence upon the bacterial activities. Wherever this fact is neglected, there is an unavoidable decline in the productivity of the land, as is demonstrated by the greatly depleted fertility of large areas in the Old and in the New World, which were formerly highly productive. The most profitable use of all organic residues, that is, barnyard manure, green manures, and crop residues should be considered very carefully. The organic substances contained therein represent food for innumerable soil organisms, and animal manure enriches the soil directly by its high bacterial content. Large applications of straw may act unfavorably because of the wide carbon-nitrogen ratio. As was discussed on p. 128, previous treatment with ammonium sulfate or urine may help to get a better balanced manure. Green manure should not be plowed under when the soil temperature is high, since the decomposition would be too rapid and great losses would result. If large quantities of it are incorporated into the soil shortly before sowing, the seed may be damaged by an abnormal growth of fungi.[1]

Under natural conditions organic residues decay mostly on the surface, and it is known that such a natural cover of humus exerts a very

[1] E. B. Fred, *Jour. Agr. Research*, vol. 5, 1916, p. 1161.

favorable influence upon physical, chemical, and biological conditions of the soil. *Mulching* with organic residues has given good results in orchards and on land used in truck farming. Where the summer temperature is high and the water evaporation strong, mulching should also be tried in the field; it keeps the soil moist and cool, and the development of the desired mellow structure is greatly facilitated. In irrigation districts much water can be saved by this practice; even weeds may serve a useful purpose in the form of mulch.

It has been tried repeatedly to prepare valuable organic manures from the inert humus accumulated in *peat* land. Chemical and bacteriological methods have been applied, but thus far without conspicuous success. Humogen, invented by W. B. Bottomley, Alphano humus, guanol, and other products have been offered by the trade. They have proved useful in gardens and greenhouses, but they cannot be recommended for general farming purposes.

Influence of the Application of Mineral Fertilizers.—The *phosphate* requirement of many soil organisms, especially of the nitrifying bacteria and of Azotobacter, is distinctly greater than that of the cultivated plants. If the soil is well supplied with phosphates, greater action may be expected from those organisms, and it seems possible that upon this behavior tests may be based which would permit a fairly accurate judgment concerning the quantities of available phosphates present in a soil. The reaction toward *potassium* is much less pronounced, although sometimes quite noticeable.

Nitrate and *ammonium sulfate* act unfavorably upon nitrogen fixation if they are used in very large quantities; but the relatively small amounts generally applied are without any effect or act even favorably. Nitrate and ammonium sulfate influence the soil reaction very much, especially if one or the other is used exclusively for a long time. Regular applications of sodium nitrate tend to increase the alkalinity of the soil, while ammonium sulfate makes the soil acid and causes fungi to grow in abundance.

Effect of Lime and of Sulfur.—An almost neutral soil reaction, not far from pH = 7, is generally best for cultivated plants and for soil organisms; but exceptions do occur. Certain cultivated plants prefer a distinctly alkaline soil, while others display the opposite behavior. Too much alkali creates unfavorable chemical and physical conditions in the soil, which can be improved by the use of acids or acid salts, as well as by strengthening the bacterial acid formation by the application of organic manures or of sulfur.

The presence of Azotobacter and its tendency to grow in laboratory tests weakly or vigorously has been recommended as a basis for classi-

fying soils according to their lime requirement and general productivity. The results obtained have been fairly satisfactory, but it seems as if the simpler determination of the hydrogen-ion concentration furnishes approximately the same information. Undoubtedly, the careful control and the adjustment of the soil reaction will gain in importance, after the influence of this factor upon plant growth and bacterial activity has been investigated more thoroughly.

Excessive liming should be avoided, because the intensive stimulation of the biochemical processes may deplete the fertility of the soil very seriously. Temporarily the germ content of a field that was heavily supplied with slaked lime may show a decrease, due to the caustic action of the lime, but an increase in number and activity will always follow.

Influence of Cropping.—Undoubtedly the microflora of the soil is greatly influenced by the crops growing on the land, but not much is known at present about these correlations. Legumes have generally been found to act most favorably, and, as a rule, the cultivated crops (potatoes, corn, beets) stand next to them. Other plants, for instance mustard, seem to display a distinctly detrimental influence upon nitrification and other desirable bacterial activities in the soil. The growing plants act upon the microflora of the soil directly and indirectly. Their roots are always covered by associations of microorganisms, and the whole area occupied by the root system is supplied with organic substances originating in living and in dying cells, which favor the growth of specific groups of bacteria. Furthermore, the root activity changes more or less the soil reaction, the concentration of the soil solution, the solubility of inorganic and of organic soil constituents, the physical structure, and the water content of the soil. All these influences together must necessarily modify the bacterial activities in the soil.

The physical, chemical, and biological conditions of a soil are most likely to be harmed by the continuous growing of one or a few crops on the same land. For maintaining the fertility of the soil it is much better if mixed crops are grown, as on meadows and pastures, or a proper crop rotation is followed. If soil is carefully tested after such different crops as legumes, potatoes, or wheat have grown upon it, it is usually not very difficult to find out that its structure as well as its bacteriological qualities are best in the first and worst in the last case. It is a good practice, now widely adopted in Europe, to grow cereals not oftener than every second year on the same field in regular rotation with legumes, potatoes, sugar beets, hemp, and other noncereals. Economic and climatic conditions do not permit the general

adoption of such a practice in America, but it is fortunate that corn evidently does not act as unfavorably upon the soil and its flora as do wheat, oats, and barley, provided it is not planted too frequently on the same field. Furthermore, in American farming more leguminous plants can be used as catch crops and with better results than in Europe, thus alleviating the disadvantageous effects exerted by a long succession of cereal crops.

Influence of Fallowing.—If the climate is not too extreme and crops are grown in proper rotation, it is generally possible to produce a profitable crop every year without leaving the field from time to time in fallow for a full year; but if the soil is very raw and the climatic conditions so unfavorable as not to permit a careful working of the ground in spring or in fall, an occasional fallowing will greatly improve the physical and bacteriological conditions of the soil. In semi-arid regions lack of water may make it impossible to raise a profitable crop every year, and here the fallow may help to secure a better structure of the soil and to store so much water in it that the soil organisms as well as the second year's crop are better supplied. In earlier times the fallow was considered to be the only reliable means of getting soil under humid conditions in good tilth; but at present it is well known that this result can be reached, as a rule, by less expensive methods.

Soil Disinfection.—Another exceptional means of regulating the soil flora is the so-called soil disinfection, which, contrary to the treatments thus far discussed, is destined to act directly upon the organisms in the soil. It needs hardly to be emphasized that a real soil disinfection is neither possible nor desirable. Merely a partial disinfection, a thorough change in the microflora of the soil is intended and can be realized. Numerous physical and chemical methods have been tried or are in use for these purposes.

The burning of field and forest soils, as well as of peat land, practiced since ancient times, and the more modern treatment of greenhouse soil by steam or hot water represent the physical methods available; their biological effects are supplemented or even surpassed by the resulting changes in the physical structure of the burned or steamed soil.

Many chemical substances have been tried for soil disinfection in greenhouses and in the field, but only a few are of practical value. These are carbon bisulfide, formaldehyde, and toluol. Figure 63 shows how the growth of grapevines was improved by such a treatment in a soil which had become "sick" because of long continued use for growing grapes. Other substances which have been tried more or

less successfully are chloride of lime, arsenic and arsenate, sulfates and other salts of iron, copper, manganese, and zinc, ether, benzene,

FIG. 63.—Effect of soil disinfection, after Russell and Petherbridge (*a*) sick soil, (*b*) same soil steamed, (*c*) treated with formaldehyde.

xylol, and different phenol preparations. Slaked lime applied in large quantities exerts a similar effect.

A great reduction in the number of organisms living in the soil

is the first marked result from all these treatments. As far as these organisms are pathogenic to plants or detrimental to the useful microflora of the soil, their destruction is of great benefit. The normal soil bacteria always show recovery after a few weeks, and because they are freed from such enemies as the soil protozoa, they multiply very rapidly making use of the substances present in the killed organisms and liberated by the heat or by chemical action from the inert material in the soil. Especially the solubility of the soil nitrogen is, as a rule, greatly increased. Occasionally unfavorable results are observed which

FIG. 64.—Cultures for legume inoculation ($\frac{1}{6}$ nat. size).

are caused by acids or other compounds freed in the accelerated decomposition of humus. Liming checks the first effect, but not the second one.

It is known that minute quantities of otherwise poisonous substances may act as stimulants upon higher and lower organisms, therefore, the last remnants of the substances used for soil disinfection may act in this manner; but this stimulating effect can not be of very great importance, since steaming improves the productivity of many soils almost in the same manner as does a treatment with formaldehyde or other chemical substances.

Soil Inoculation.—For enriching the soil with useful organisms barnyard manure and compost have been used since ancient times, but not before Pasteur had directed the general attention to these biological problems, has it been known that the beneficial effect of such a treatment is at least partly due to the high germ content of these manures. It is not to be denied that some experiments have given and will give results which apparently do not support this statement; but a marked

FIG. 65.—Serradella (Ornithopus sativus) (*a*) not inoculated, (*b*) inoculated ($\frac{1}{3}$ nat. size)

improvement of the biological soil conditions has frequently been recorded, confirming the practical experiences that stable manure and compost are the best means of getting "life" into the soil.

Pure cultures or mixed cultures of certain soil organisms, especially of nitrogen fixing bacteria, have been tried for soil inoculation. A few promising and many unsatisfactory results have been obtained. If it is kept in mind that the bacteria or their spores or gonidia are present nearly everywhere, but that a good development can not take place except under favorable conditions, it is almost self-evident why

soil inoculation rarely succeeds. If the environmental conditions are favorable, the useful bacteria will be present in the soil; if they are missing, it is almost invariably due to unfavorable conditions, which would first have to be improved. Alinite, All Crops Soil Inoculum, Bacto Natural, U-cultures, Phosphogerm are the names of some commercial preparations which have been or are widely advertised for soil inoculation, usually under very exaggerated claims. Bac-Sul is sulfur inoculated with sulfur oxidizing bacteria, which is said to be much more rapidly oxidized in the soil, because here the specific organisms are usually present in small numbers only.

FIG. 66.—Soy beans (Soja max.) inoculated (left), and not inoculated (right).

Plant Inoculation.—If pure cultures of nodule bacteria are used for inoculating leguminous plants, good results can be expected, because the growing plant offers the most suitable environmental conditions. After the same kind of legume, or one which permits cross-inoculation, has grown repeatedly on the same field, normal nodules are produced in abundance, because the soil is gradually enriched with the special type of nodule bacteria; but if there is any doubt as to the presence of enough bacteria of the desired type, the seed should be inoculated to assure prompt nodule formation and vigorous nitrogen fixation.

Before pure cultures became available for this purpose, soil was frequently taken as inoculum from fields where a good crop of the special kind of legume had been grown. This practice is still to be recommended when no reliable pure culture can be secured, provided

the soil does not contain too many weeds and plant diseases. As a rule, however, efficient pure cultures for legume inoculation can easily be secured at present from Agricultural Experiment Stations, Agricultural Departments, and from many commercial firms. A number of them are shown in Fig. 64. Many different trade names have been invented, such as Azotogen, Farmogerm, Nitragin, Nitrobacterine, Nitroculture, Westrobac, etc. The cultures are furnished in liquid form, on agar, or in soil. Since good soil is undoubtedly the most suitable of the three substrates, such cultures give generally the best results. If the preparations are only a few days or weeks old, their quality depends exclusively on the care with which the cultures have been selected and propagated.

Successful inoculation increases the crop considerably, as may be seen from Figs. 65 and 66; the cost is very moderate. Growing of inoculated legumes is one of the best means of maintaining and increasing the productivity of the soil.

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