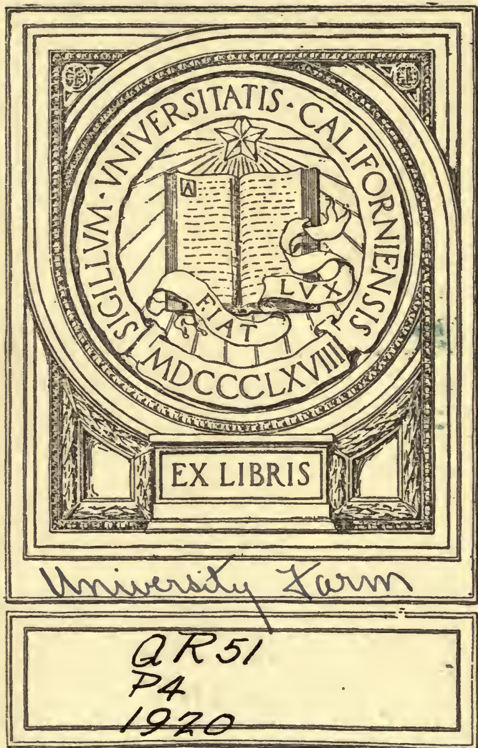
AGRICULTURAL BACTERIOLOGY

JOHN PERCIVAL



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AGRICULTURAL BACTERIOLOGY

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THEORETICAL AND PRACTICAL

BY

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SECOND EDITION

LONDON
DUCKWORTH & CO.
3 HENRIETTA STREET, COVENT GARDEN, W.C.
1920

First Edition, 1910
Second Edition, 1920

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Printed in Great Britain
by Turnbull & Spears, Edinburgh

PREFACE

THE science of bacteriology, which has been so much the domain of the student of disease, is now being applied to the elucidation and solution of many of the problems which confront the farmer, gardener, and dairyman in their daily life.

The extensive development of the dairying industry has been greatly assisted by the bacteriologist, and very material advances may be expected in the near future in the application of the science to the study of the soil and the phenomena connected with the economical and efficient use of fertilisers for the nutrition of farm and garden crops.

For some time a knowledge of bacteriology has been demanded of candidates for examinations in agriculture, dairying, and horticulture, but experience has shown that few of them have had any practical acquaintance with the subject, mainly, we believe, from lack of suitable opportunities for proper training and absence of a text-book dealing with the subject in a practical way.

It is to supply the needs of such students that the present text-book has been written. It is hoped also that it will be useful and interesting to agriculturists and horticulturists generally who are anxious to obtain an insight into the causes and methods of control of many

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of the natural processes with which they come into contact daily.

The work embodies the results of much experience in the scientific training of students, and deals with most of the points which the author considers of fundamental importance in the study of bacteriology, so far as the requirements of the farmer and gardener are concerned.

I shall be grateful for any suggestions for improving the work.

My thanks are due to Miss Grace Heather Mason for assistance in carrying out the work of the practical exercises and also for help with the index and proofs.

JOHN PERCIVAL

May 1910.

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AGRICULTURAL BACTERIOLOGY

CHAPTER I.

INTRODUCTION.

1. TAKE some hay and chop it into short pieces about an inch long. Then place it in a glass beaker or cup, pour cold water over it, and leave in a warm room for a day or two. The cold water dissolves various substances from the hay and becomes brownish in colour, rather like weak tea. If a drop of the solution is taken from the cup or beaker and examined with a microscope, many living things will be found swimming about in it. Fig. 1 gives the appearance of what is generally seen in such a drop examined under a moderate magnification (an ordinary microscope with a $\frac{2}{3}$ objective). The most obvious living beings are small oval transparent organisms which glide in all directions across the field of view (Fig. 1, *a*). Sometimes there are few of them, but in the majority of trials with a hay infusion they will be met

FIG. 1.—Drop of hay infusion. *a* Protozoa; *b* bacteria (×200).

with in considerable numbers, and frequently several kinds are visible. These all belong to the Animal Kingdom, and are included in the Protozoa, a division which embraces the simplest forms of animal life.

Small pieces of leaves may also be observed in most instances, as well as other bits of dead material. In addition to these there will always be visible hundreds of exceedingly small rod-shaped bodies distributed about in the water. They are so minute, however, that unless the attention is specially directed to them they may easily be overlooked. By careful observation they are seen to be of variable lengths and capable of independent movement, travelling end on backwards and forwards through the liquid.

Fig. 2 is a more highly magnified view of a portion of a drop of a hay infusion, an $\frac{1}{8}$-inch objective being used in this case. The large oval body, *a*, is one of the Protozoa referred to above; *b* is a piece of mould fungus, and some of its spores or reproductive bodies are seen at *c*. They are non-motile. The living, rod-like organisms are now easily seen, and their movements more readily followed. Some of the longer ones are depicted in the figure at *d*, and others of a different form and size are shown at *e*.

2. These minute living things are examples of organisms known as *bacteria*. They are distributed all over the world in incalculable numbers—in the air, in water, in the soil, and wherever dust can be carried. Their importance can scarcely be over-estimated, as they are responsible for some of the most striking phenomena in nature. They carry on useful work in preparing the soil for the growth of crops, and render manures which are applied to the land efficient for the nutrition of

plants. They act as natural scavengers, getting rid of all kinds of dead organic matter through the process of fermentation and decay which they initiate and maintain. Several industries, such as the manufacture of butter and cheese, could not flourish without their aid. On the whole their action is beneficial. Certain kinds are

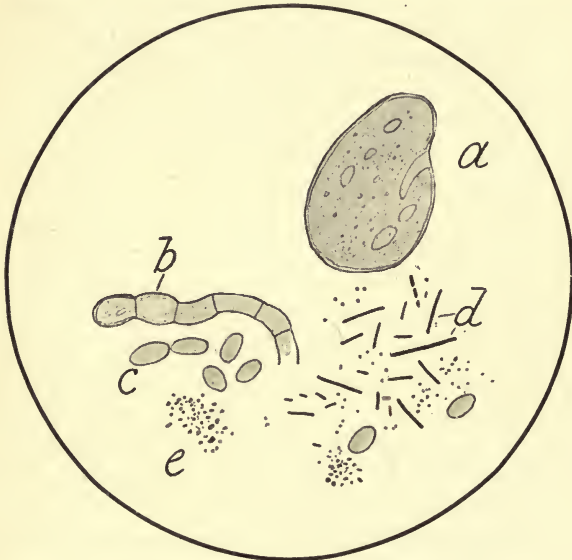


FIG. 2.—Drop of hay infusion. *a* Protozoon ; *b* hypha of a fungus ; *c* spores of fungus ; *d* and *e* bacteria. (700).

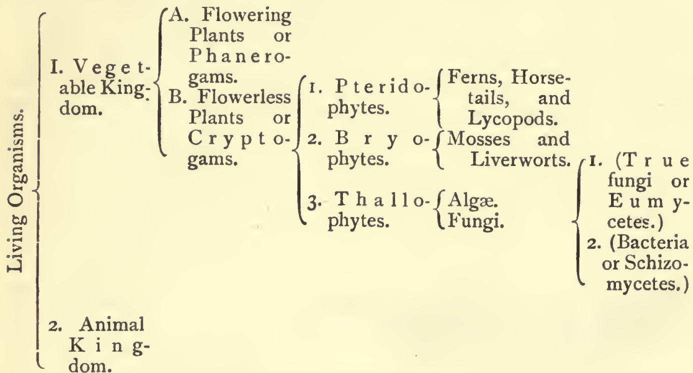
however, the cause of some of the most fatal diseases affecting man and the lower animals.

3. **Relationship of these Organisms to others in the Vegetable Kingdom.**—As bacteria are generally spoken of as minute unicellular plants, it is of interest to indicate the position in which they are usually placed among the divisions of the Vegetable Kingdom.

Living things are generally classified into plants and animals, and there is little difficulty in placing the higher forms, such as cows, birds, thistles, or cabbages, into one or other of these two groups. *Flowering plants* are characterized by the production of special structures which botanists call seeds; these contain rudimentary plants. *Flowerless plants*, on the other hand, never produce seeds, but grow from unicellular structures termed spores. Of flowerless plants three divisions are generally recognized, viz.: (1) Ferns and their allies; (2) Mosses and allies; and (3) Thallophytes, which differ from the others in possessing no definite root, stems, or leaves. The Thallophytes are subdivided into (1) *Algæ* and (2) *Fungi*. The *Algæ*, to which belong sea-weeds and their relatives in fresh water, possess the green colouring matter, chlorophyll, by the aid of which they can utilize the carbon dioxide of the air for the production of carbon compounds of their bodies when they are exposed to daylight. *Fungi*, on the other hand, contain no chlorophyll, and, with the exception of a small group of bacteria, they are unable to make use of carbon dioxide for the formation of their carbon compounds. They require to be fed with organic food, such as starch, sugar, fats, proteins, and other similar substances directly derived from other living or dead plants and animals.

The true fungi or *Eumycetes*, of which some common examples are described in Chapter XXV., generally consist of branching, thread-like cells, and are reproduced by spores. Bacteria are usually associated with this division of the Vegetable Kingdom, chiefly because of their want of chlorophyll and general mode of nutrition, but in other respects they exhibit little relationship with the true fungi, and their real position in a natural scheme of classification,

based upon what may be termed blood relationship or descent, is uncertain. However, for practical purposes this is of little moment, and they may be left as a division of the Fungi, as indicated in the following scheme :—



Ex. 1.—To clean Slides and Cover-Slips.—Before any satisfactory microscopic work can be done, it is necessary to clean thoroughly all glass slides and cover-slips or cover-glasses.

(1) Place them in a hot solution of caustic soda (caustic soda about 10 grams, water 100 c.c.) for an hour.

(2) Take them out and wash under water-tap: wipe and dry them.

(3) Place them, one by one, in a beaker containing strong nitric acid, and leave them for one or two hours.

(4) Take them out, wash under tap, wipe and dry with a clean handkerchief.

(5) Store them in a beaker or specimen jar containing absolute alcohol until wanted. When required for use, take out and wipe clean.

Ex. 2.—Heat a platinum wire loop (Fig. 3) in a Bunsen flame; dip it just below the surface of an infusion of hay which has been prepared in the manner described above and left in a warm room

or incubator for four days. Transfer the drop on the wire loop to a clean slide: put on cover-glass and examine with low power, and then with $\frac{1}{8}$-inch objective. Make drawings of bacteria seen.

Before and after using a platinum loop or needle always heat it to redness in Bunsen flame: when not in use keep it on a stand (Fig. 3); do not place it on the laboratory table or bench.

Ex. 3.—Make infusions of the leaves of cabbage, cereals, clover, and other plants. Pour off the liquid, after it has been on the leaves for three or four hours, into a clean beaker. Leave in a warm room for four days, and examine drops as in preceding exercise. Make drawings of bacteria seen.

Ex. 4.—Take some peas and soak them in water all night. Then boil them for five minutes, and leave them to stand in the boiled water for a day or two in a warm room. Take a drop of the liquid and examine for bacteria.

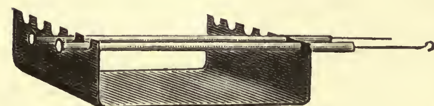


FIG. 3.—Copper stand for platinum needles and loops.

Ex. 5.—Staining of Bacteria.—Since bacteria are transparent and not readily seen, they are frequently killed and stained, when their form is rendered more easily visible.

(1) Prepare a saturated solution of **gentian violet** in alcohol, using

Gentian violet	4 gr.
Absolute alcohol	50 c.c.

Shake well, and allow it to stand twenty-four hours; then filter twice into a stoppered bottle; label it, and keep for future use.

(2) Add three drops of the above alcoholic solution to a watch-glass containing distilled water.

(3) Take single drops of the infusions from Exs. 3 and 4 and spread them with the platinum loop as thin *films* over the surface of clean cover-glasses. Cover with a beaker to keep off dust, and allow the films to dry. The moisture evaporates and leaves the bacteria behind on the glasses.

(4) Hold the cover-glass with Cornet forceps (Fig. 4) and fix the bacteria by passing the glass, *bacteria side up*, three times through Bunsen flame. In doing this the hand should describe a circle about one foot in diameter, at the rate of once round every second, the path of the glass passing through the flame.

(5) Place the cover-glass, *bacteria side down*, on the surface of the watery solution in the watch-glass (2), and allow it to remain there for three to five minutes, during which time the bacteria will become stained. Wash it in water and dry between filter paper.

(6) Clean the side of the cover-glass on which there are no bacteria, and place it on a drop of water on a clean slide. Examine and make drawings of the stained bacteria.

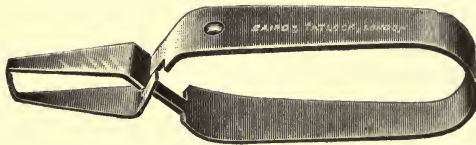


FIG. 4.—Cornet spring forceps.

(7) If not sufficiently stained, float off the cover-glass with water and stain again; re-examine it after washing with water, and, if satisfactory, dry it again between filter paper and leave it in a warm place until all traces of moisture are gone; then mount it permanently in a drop of xylol balsam (Canada balsam dissolved in xylol).

Ex. 6.—Prepare a solution of **methylene blue** in alcohol in the same manner as the gentian violet solution in Ex. 5, using:—

Methylene blue	4 gr.
Absolute alcohol	50 c.c.

Label it and store in a stoppered bottle. Try its staining power upon cover-slip preparations obtained as in Ex. 5 (3).

Ex. 7.—Prepare the following solution :—

Löffler's Methylene Blue.

(1) Make up a 1 per cent. solution of caustic potash.

(2) Take 1 c.c. of it and add it to 100 c.c. of distilled water.

(3) Mix weak solution (2) with 30 c.c. of a saturated alcoholic solution of methylene blue (Ex. 6). Shake, filter into a stoppered bottle, and label it.

This is an excellent stain, not very deep, but it often brings out delicate features of bacteria; it loses some of its staining power on keeping. Try it on cover-glass preparations as described in Ex. 5, allowing it to act for 3-10 minutes.

Ex. 8.—Prepare the following solution :—

Ziehl-Neelsen's Carbol-fuchsin.

Fuchsin	1 gr.
Absolute alcohol	10 c.c.
Carbolic acid (crystals)	5 gr.
Distilled water	100 c.c.

Dissolve the fuchsin in the alcohol in a mortar or porcelain dish, breaking the fuchsin if necessary with a pestle or a glass rod. Then dissolve the carbolic acid in the water and add half of this solution to the dye in the mortar or dish; stir well, and pour into a stoppered bottle. Wash out the mortar with the rest of the carbolic-acid solution, adding it to the mixture in the bottle. Shake well, and after twenty-four hours filter it into an amber-coloured stoppered bottle or ordinary glass bottle covered with brown paper to exclude the light.

This gives an intense red stain. It is best to use a freshly prepared weak solution made by adding 1 c.c. of the above concentrated solution to 10 c.c. of distilled water; filter. Try it on cover-glass films as described in Ex. 5, allowing it to act for 1-3 minutes.

Ex. 9.—Gram's Method of Staining.—Prepare the following solutions :—

(*a*) Saturated alcoholic solution of gentian violet, 30 c.c.
Aniline water, 100 c.c.

As this solution does not keep well, only a small amount should be made up.

The aniline water is obtained by thoroughly shaking up 4 c.c. of aniline with 100 c.c. of water, and then filtering through moist filter paper until the liquid is clear and free from any oily drops; any aniline which remains in the bottom of the flask or tube after shaking should not be poured on the filter.

(*b*)	Iodine	1 gr.
	Potassium iodide	2 gr.
	Water	300 c.c.

Dissolve the potassium iodide in about 10 c.c. of the water and add the iodine: when the latter is dissolved add the rest of the water.

In using the above solutions for staining purposes, the following procedure should be adopted:—

(1) Float the cover-glass, *film downwards*, on aniline-gentian-violet solution in a watch-glass, and allow the stain to act for five minutes.

Take up with forceps and drain off the superfluous stain by dipping the edge of the cover-glass on blotting-paper.

(2) Then, immerse the cover-glass for 1-2 minutes in the iodine solution in a watch-glass, *film side up*. The purple colour of the gentian violet should change to a yellowish brown tint.

(3) Now, remove the cover-glass, taking care to note on which side the bacteria are; drain off the superfluous liquid and dip the cover-glass into a vessel containing absolute alcohol for 10 seconds, and into a second vessel of absolute alcohol for 10-15 seconds; when no more of the gentian violet stain is seen to wash off, take it out and wash it in water, and examine either in water or after drying in Canada balsam. Too long immersion in alcohol must be avoided.

Bacteria may be divided into two groups, viz.:—

i. Those which stain by Gram's method (Gram positive, +)

ii. Those which do not retain the gentian-violet stain when washed a short time with alcohol . . (Gram negative, −)

In using the test upon a doubtful organism of *coccus* form, it is sometimes useful to mix the latter with a *bacillus*, such as *Bs. subtilis*, known to stain by Gram's method ; or, if a *bacillus* is to be tested, mix it with a Gram-staining *coccus*, such as *Str. lacticus*.

The following organisms are **Gram positive** :—

Most species of *Micrococcus*.
" " *Sarcina*.
Bs. subtilis.
Bs. megatherium.
Bs. mesentericus.
Bs. mycoides.
B. (Proteus) mirabilis.
B. (Proteus) Zenkeri.
Bact. acidi lactici.
Str. lacticus.
Most yeasts and fungi.

The following are **Gram negative** :—

Most species of *Spirillum*.
Bact. coli.
Bact. lactis aerogenes.
Bact. fluorescens.
Bact. prodigiosum.
Bact. pyocyaneum.

Try Gram's stain upon a series of cover-glass films of organisms obtained from the hay, cabbage, and pea extracts of Exs. 3 and 4.

CHAPTER II.

BACTERIA: THEIR FORMS AND REPRODUCTION.

1. **The Forms of Bacteria.**—The body of a bacterium consists of a single cell, into the minute structure of

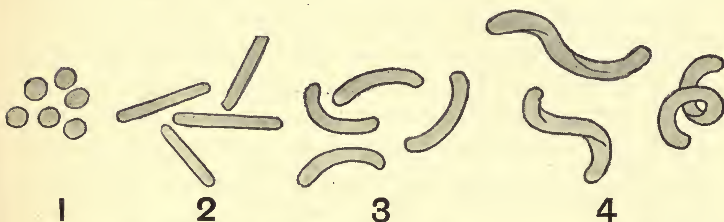


FIG. 5.—Forms of bacteria. 1, Coccus; 2, bacillus; 3, vibrio; 4, spirillum.

which it is not necessary to enter here. It is sufficient to note that there is a *central body* of protoplasm which stains readily with aniline dyes of various kinds, and a skin or *capsule*, usually so thin as to be practically invisible except by the aid of the highest powers of the microscope. The capsule, or cell-wall, is not composed of cellulose as in the higher plants, but of a material apparently allied to the protein substances. The whole body or cell is met with in many forms, as indicated in Fig. 5. Some kinds of bacteria are spherical, each individual being then termed a *coccus*. When much longer than broad, the bacterium is known as a *bacillus*; a comma-shaped form is spoken of sometimes as a

vibrio; a rigid spiral form in shape like a corkscrew is a *spirillum*.

The terms *bacterium* and its plural *bacteria* are frequently used in a general sense when speaking of one or more or any of these minute organisms without reference to their form. The word *bacterium* is, however, used also in a limited sense to denote a particular non-motile rod-like cell which is shorter than a bacillus. Under certain circumstances bacterial cells may become distorted and irregular in shape. Such are known as *involution-forms* (Fig. 6), and are met with chiefly where the medium in which they are living is becoming exhausted of its nutritive substances, or where there is an accumulation of compounds which are detrimental to the vitality of the organisms: they are diseased, degenerate forms.

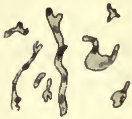


Fig. 6.—Involution forms of a bacterium

The capsule or skin surrounding the central body of a bacterium as a rule is very thin, and as it does not readily absorb dyes it is rarely visible. In some species, however, the capsule is of a mucilaginous or slimy nature, and swells up with water to a thickness of several times that of the rest of the cell (Fig. 7). It may then be faintly stained in several ways, and can be seen as a pale cell-wall surrounding the darker central body of the bacterium. Very frequently the swollen envelopes of large numbers of bacteria adhere to each other, and form a gelatinous mass or *zooglœa* colony of variable size, which is often met with as a slimy skin-like "scum" on

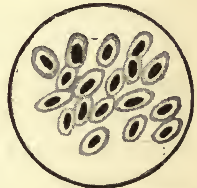


Fig. 7.—Bacteria, showing well-developed capsules.

stagnant water, or in solutions containing decayed organic matter.

2. **Size.**—Bacteria are among the smallest of all living things. Their dimensions are usually stated in thousandths of a millimetre. One thousandth of a millimetre (.001 mm.), known as a *micron*, is denoted by the sign 1 μ, and is equal to about $\frac{1}{25000}$ of an inch (.00004 inch). Cocci range from less than .5 μ up to 2 μ in diameter; bacteria and bacilli from .5 μ or less in breadth to 10 μ in length; the majority, however, are much shorter than the latter figure. On account of their minute size they are, when dry, readily blown about by the slightest breeze, and find their way into any crevices wherever dust can penetrate. They are carried in streams of water, and are to be found in and about all foods, milk and other liquids, in houses and stables and in the soil, on the skin and in the stomach and intestines of all animals. They are, however, never met with in the blood or tissues of healthy animals or plants, and are rare in water which has come from deep wells or springs, or has been filtered through great depths of soil and rocks.

Ex. 10.—To Measure the Size of a Bacterium or other Organism.

(*a*) A simple method is by means of a stage micrometer and camera lucida.

The stage micrometer consists of a scale of tenths and hundredths of a millimetre accurately ruled in fine lines on a glass plate.

Place it on the stage of the microscope, and make drawings of its scale on a sheet of paper with the camera lucida, using in turn the $\frac{2}{3}$ in., $\frac{1}{6}$ in., $\frac{1}{8}$ in., and $\frac{1}{12}$ in. objectives, with a No. 3, 4, or other eyepiece.

Take away the stage micrometer, and in its place put the slide of the bacterium to be measured; make a drawing of the organism with the camera lucida. Now measure the dimensions of the drawing with the scale prepared as above from the micrometer scale, taking care that the eyepiece and objective used in making the paper scale and the drawing of the bacterium to be measured are the same.

(i) Measure any bacteria which you have mounted and stained.

(ii) Mount a little yeast in water and measure the size of the cells.

(iii) Measure the spores of any mould which you can obtain.

(*b*) A bacterium or other organism may be measured directly by means of an eyepiece scale and a stage micrometer. The eyepiece scale consists of a disk of glass, which fits into the interior of the eyepiece; upon it a fine scale of equi-distant lines is ruled.

Place the micrometer, ruled in $\frac{1}{10}$ths and $\frac{1}{100}$ths of a millimetre, on the stage of the microscope, and examine it with the eyepiece having the scale in it.

Bring the divisions of the latter into line with those of the stage micrometer, and find the value of one division of the eyepiece scale in $\frac{1}{10}$ths or $\frac{1}{100}$ths of a millimetre.

Use the various objectives successively, and record the results for each.

Place the bacterium or other object to be measured on the microscope stage and determine how many divisions of the eyepiece scale it covers: it is now easy to calculate its real dimensions, as the value of each division of the eyepiece scale is known.

(i) Measure the objects mentioned above in (i), (ii), (iii) of section (*a*) in this way, and compare the results obtained by the previous method.

3. **Movement.**—In the first chapter it was noticed that some bacteria are endowed with the power of spontaneous movement; they can rove from one part of a drop of water to another as readily as fish in a pond. This is brought about by the lashing action of one or more exceedingly delicate hair-like *cilia* or *flagella* which are attached to the outer coat or capsule of the bacterium. Some organisms possess only one flagellum at one end (*monotrichic*), others two or more in a tuft at one or both ends (*lophotrichic*), while many bacilli have many flagella distributed all over the cell-wall, and are spoken of as *peritrichic* (Fig. 8). Numbers of species of bacteria are, however, devoid of motile cilia, and are incapable of spontaneous movement in liquids. Very frequently non-motile forms exhibit a peculiar oscillatory movement when put in water or other liquids. This motion backwards and forwards over a very short distance or round and round in a circular or oval path is a purely physical phenomenon known as Brownian movement, and is exhibited by any kind of exceedingly small particles, either living or dead, when they are suspended in water.

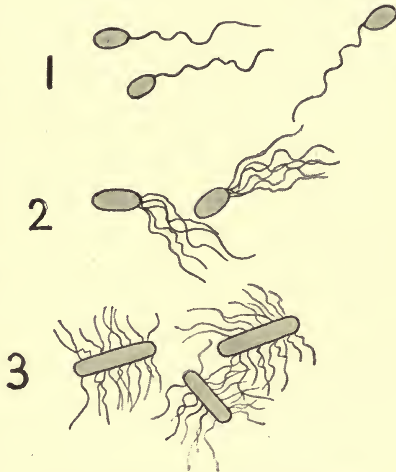


FIG. 8.—Arrangement of flagella on bacteria. 1, Monotrichic; 2, lophotrichic; 3, peritrichic.

Ex. 11.—Staining of Flagella.—The flagella or motile organs of bacteria are of extraordinary fineness and delicacy, and appear to dissolve or become thrown off and disorganized when the water in which the bacteria are living dries up.

Under certain conditions they may be rendered visible by staining, but the operation is one of much difficulty and uncertainty. The chief elements of success are extreme cleanliness of the cover-glasses on which the organisms are placed, and a culture of the bacteria in the right state of development, the best results being obtained from growths eight to sixteen hours old upon an agar medium.

Various staining methods have been devised, those of Loeffler and Van Ermengem being as useful as any.

Loeffler's Method.

(1) Take an absolutely clean slide, cleaned as in Ex. 1; wipe it with a clean handkerchief, and handle it with the fingers as little as possible. Place upon it three small drops of water.

(2) With a fine platinum loop remove a small portion of a six to eighteen hours old agar culture of *Bs. subtilis*, *Bact.* (*Proteus*) *vulgare*, or other motile species, and place it in the first drop of water: this will become slightly turbid. Now pass the platinum loop through the flame, and, when cool transfer a part of the first drop to the second on the slide; in the same way add a small part of the second to the third drop. By diluting in this manner the bacteria become separate from each other.

(3) Take one or two absolutely clean cover-glasses. Now dip the end of a platinum needle, the top of which has been bent at right angles, into the third drop, and with one stroke gently rub the water which adheres to it on to the surface of the cover-glass; repeat the operation on another part of the cover-glass. Dry the cover-glass in an incubator at 37° C. for three or four minutes. The dilutions, spreading of the drops and smears, and

the drying of the latter should be carried out as rapidly as possible.

(4) Place the cover-glass in Cornet forceps, and cover the surface of the film with a few drops of the following mordant, which must be filtered :—

A 20 per cent. solution of tannin . . .	10 cc.
Saturated solution of ferrous sulphate . .	6 ,,
Saturated alcoholic solution of fuchsin . .	1 ,,

Warm the cover-glass, and allow the mordant to act for four to six minutes; then wash with water from the tap, and before the glass dries cover the surface with the following stain :—

Fuchsin saturated alcoholic solution . .	6 cc.
Aniline water (see Ex. 9)	20 ,,

Warm, and allow the stain to act for three minutes.

(5) Now wash the cover-glass with water; dry it in an incubator at 37° C.; mount it in a drop of xylol, and examine with a $\frac{1}{12}$ in. objective.

Van Ermengem's Method.

(1) Prepare the cover-glass films as in (1), (2), and (3) above.

(2) Place the cover-glass films in the following solution, which must be three or four days old :—

Osmic acid 2 per cent. solution . . .	10 cc.
20 per cent. tannin solution	20 ,,
Glacial acetic acid	1 drop

Allow the solution to act for one hour at room temperature, or five minutes at 50° to 60° C.

(3) Wash first in distilled water, then in absolute alcohol, and finally in distilled water again.

(4) Dip the cover-glass for thirty seconds in a .5 per cent. solution of silver nitrate; move it about gently in the liquid.

(5) Drain off the superfluous liquid, and then dip the cover-glass for thirty seconds in the following solution:—

Gallic acid	5 gr.
Tannin	3 ,,
Fused acetate of soda	10 ,,
Distilled water	350 cc.

(6) Drain and dip it again in the silver nitrate solution (4), moving it about until the film becomes blackish.

(7) Now wash it thoroughly in distilled water; dry it; when quite dry mount it in xylol balsam, and examine with a high power.

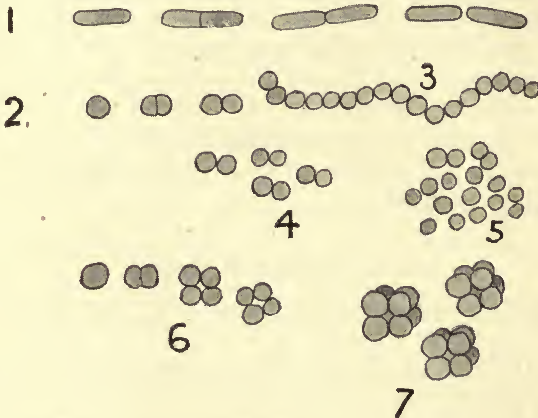


FIG. 9.—Vegetative reproduction of bacteria.
1. Bacillus with successive divisions.
2. Coccus giving rise to chain 3 (*Streptococcus*), pairs 4 (*Diplococci*), irregular group 5 (*Staphylococci*).
6. Coccus dividing in two directions (*Micrococci*).
7. Coccus dividing in three directions (*Sarcinæ*).

4. **Reproduction.** (i) **Vegetative Reproduction.**—When bacterial cells reach their adult normal form if the conditions of temperature and nutrition are satisfactory, a partition or septum is developed across the cell which divides it into halves. The two halves, or daughter-cells, then grow to an adult size and undergo similar division (Fig. 9). The process is repeated, and vast numbers

may arise in this way. It is this peculiarity which has led to the name Schizomycetes, which means splitting or fission fungi, being applied to this group of organisms.

The dividing partition in the case of elongated forms of bacteria is placed across the shortest diameter. Very frequently several generations produced by such division remain attached to each other instead of separating into individual, isolated bacteria. Many round or coccus forms divide regularly in one direction and give rise to longer or shorter chains included under the term *streptococci*. Cocci occurring in pairs are termed *diplococci*. Sometimes the divisions are irregular although in one plane, and groups are produced which are often spoken of as *staphylococci*. In some cocci division of the cell into four daughter cells occurs by two partitions at right angles to each other; such are termed *pediococci*. In other kinds separation into eight cells takes place by division in three directions of space, and as in this class the cells of successive generations usually remain attached to each other, cubical packets or bundles of cocci are produced; they are classed under the name *sarcinæ* (Fig. 9). As previously stated, bacteria and bacilli which are longer than broad in the majority of cases separate by division plates which are placed across the cell transversely. If the cells produced remain attached, long thread-like filaments are formed (Fig. 10). Longitudinal division also occurs among bacteria, but only very exceptionally.

FIG. 10.—Chains of bacilli.

The rate at which vegetative reproduction takes place

is dependent upon temperature, air supply, nutrition, and other circumstances, but under favourable conditions division may occur once in every hour or less, so that from one individual many millions of descendants can be produced in a day when placed in milk or other suitable nutritive liquids.

(ii) **Reproduction by Spores.** — Under certain conditions, and especially those which lead to unfavourable or unhealthy growth, such as unsuitable temperature, dryness, excess or deficiency of air supply, exhaustion of nutrient materials and accumulation of waste products, the vegetative division of bacteria is retarded and stopped. The protoplasm within the cell contracts and forms an oval body known as a *spore* or *endospore* (Fig. 11), which, on account of its high refraction, appears as a bright glistening spot surrounded by an apparently empty shell of the mother-cell. The latter sooner or later swells up and dissolves, leaving the free spore behind. The protoplasm of the spore is denser than that of the original mother-cell, and the coat of the spore is a somewhat thick membrane which is not readily permeable to dyes or other solutions. The number of spores which originate in each bacterium is generally one, though in one or two kinds two or more spores are seen in each cell. The spore occupies various positions in the cell arising in some species at one end, while at others it is formed near the middle of the bacterium. In the genus, *Clostridium*, the mother-cell assumes a peculiar spindle-shaped form, swollen in the middle and narrow at the ends when spore formation takes place: it may also be shaped like a drumstick as in the bacilli which cause tetanus (Fig. 11). In some species of bacteria endospores are not found, but their vegetative cells cease to divide, be-

come surrounded by a thick wall and assume the structure and character of spores; these are designated *arthrospores*. The spores of bacteria are capable of resisting adverse conditions which would instantly destroy the vitality of the organisms in an active vegetative state. Some of them can withstand boiling in water for an hour, and are not damaged by chemical solutions which would kill off all growing bacterial cells. They are also able to retain their power of germination for many years in a

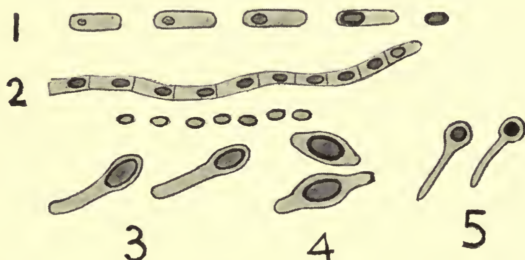


FIG. 11.—1. Spore formation in a bacillus.
2. Chain of bacilli with spore in each cell; isolated spores below.
3 and 4.—Forms of *Clostridium* cells with spores.
5. Drumstick-shaped cells with spores.

dry state. When placed under favourable conditions of temperature, and provided with a suitable moist nutrient material and a proper air supply, the spores germinate. They absorb water, enlarge and become less refractive. In the anthrax bacillus the whole spore becomes gradually transformed into a new elongated bacterium or bacillus, the thin spore membrane is absorbed in the surrounding liquid, and the protoplasm gradually assumes the form of an actively-growing vegetative rod. In *Bs. megatherium*, after the spore has absorbed a certain amount of water its cell wall opens at one end and the protoplasmic contents escape and grow into a typical rod-

shaped bacillus. This is spoken of as *polar* germination. In *Bs. mycoides* the protoplasm of the spore grows out through an opening which arises in the side of the spore (*equatorial* germination), the time taken for complete germination being in many cases about three or four hours at a temperature of 30° C. (Fig. 12).

FIG. 12.—1. Equatorial germination of spore.
2. Polar germination of spore.

Ex. 12.—Staining of Spores.—Prepare cover-glass films of "scum" from an old hay decoction (par. 1, Chap. I.) and from other old cultures likely to contain bacteria with spores. Fix by passing through flame three times. Wash for two minutes in chloroform to get rid of any drops of fat and other materials. Wash with water and dry with filter paper. Pass through Bunsen flame again.

Place now in a 5 per cent. solution of chromic acid for twenty to thirty seconds (in some cases ten seconds is enough, in others better results are obtained if left for two to three minutes or longer; further trials must be made if twenty to thirty seconds give poor results). Wash in water. Dry and fix again.

Place now in carbol-fuchsin (Ex. 8) and heat for three minutes. Wash with water and dip into 5 per cent. sulphuric acid for five seconds to get rid of the fuchsin from all parts of the bacteria except the spores. Wash again in water and then stain for a minute with Loeffler's methylene blue (Ex. 7). Each spore will be stained red and the rest of the bacillus blue.

Ex. 13.—Apply films to cover-glasses, leave to dry in air without heat. When dry, place in hot ½ per cent. hydrochloric acid for three to four minutes. Wash with water, dry and fix in flame. Then stain with carbol-fuchsin; wash in acid and counter stain with methylene blue as in above Exercise.

Ex. 14.—Observe the **germination of spores** on an agar "hanging block."

Take 10 c.c. of melted agar medium and pour it into a sterile Petri dish about 4 inches in diameter. After cooling cut out of it some square pieces of the solidified agar about 8 to 10 mm. across with a sterile knife. Lift up a piece or block and place it on a sterile glass slide; cover it with a bell jar to keep off dust until wanted.

Take a small amount of a culture containing spores from agar or other medium and mix it with 1 c.c. of sterile broth in a sterile test tube; heat the mixture for ten minutes in a water bath at 75° C. to kill all growing organisms; the spores remain uninjured. Spread a small loopful on the surface of the agar block and cover with a thin sterile cover-slip. Now run a needle under the lower surface of the agar and lift the cover-slip with the block attached to it; place it over a culture cell (made by cementing a rubber, metal, or cardboard ring to an ordinary slide with Canada balsam or other cement) so that the agar block hangs down into the cell.

Before putting on the cover-slip a small amount of vaseline may be smeared round the edge of the cell to prevent evaporation of the agar medium.

Place the whole now in a large Petri dish in an incubator at 25° to 30°. Make observations of the germination of the spores and subsequent growth of the bacteria through the cover-slip at intervals of three to six hours.

Ex. 15.—Try and observe the germination of the spores of bacteria introduced into sterile broth as above, a drop of the mixture being arranged as a hanging drop as figured and described in Ex. 35.

5. **Classification of Bacteria.**—The bacteria form a class of the sub-kingdom Schizophyta or Fission-plants, thus:—

Sub-Kingdom. *Schizophyta.*

Class I. *Schizophyceæ* or Blue-green Algæ.

Class II. *Schizomycetes* or Bacteria.

The former class usually contains chlorophyll or an allied colouring material, and its representatives are very commonly distributed in water or on moist soil, wood, rock, and stones, where they form patches, usually of a green or bluish-green colour. Schizomycetes or bacteria resemble the Schizophyceæ in the simplicity of their organization, but contain no chlorophyll, except possibly in a few instances. Many species, however, have the power of forming purple, pink, yellow, and other colouring material, and are not uncommon on decaying meat, bread, and other organic substances, where they form red, purple, or yellow spots.

The grouping of bacteria into families, genera, and species is a matter of great difficulty. Various methods have been proposed, based on the forms of the cells, the presence and absence of spores, the possession or want of flagella, and the power of producing disease and setting up chemical changes. Unfortunately, a coccus may lengthen into a bacillus form, or a bacillus may develop in the form of a coccus under certain altered conditions of growth and nutrition, so that the shape of the cell cannot be used as a perfect basis of classification; nor are the presence or absence of cilia and the possession of spores very satisfactory for the purpose, since the same organism may be unable to form spores or develop cilia at one time, and yet may do so when other external conditions prevail. Many of the chief generic names of bacteria are based on the form which the organisms most commonly assume. We thus have the genera, *Micrococcus*, *Streptococcus*, *Sarcina*, *Bacterium*, *Bacillus*, and *Spirillum*, characterized by the cell-forms described on pages 11, 12, and 19. There is a tendency among some authorities to restrict the generic term *Bacillus* to rod-like bacteria

which possess flagella distributed all over the cell-wall and are motile, the genus *Bacterium* consisting of those rod-like forms which are devoid of motile organs. The presence or absence of cilia is found, however, to be a variable character, even in the same species, an organism at one time appearing to be without them, while, when grown on another medium and at a different temperature, it may become motile and possess cilia. The scheme, if rigidly carried out, would necessitate much alteration of existing names, and would be likely to lead to confusion. It is perhaps best at present to include the non-sporing, rod-shaped organisms in the genus *Bacterium*, those with genuine endospores being placed in the genus *Bacillus*. The genus *Pseudomonas* includes rod-like forms with flagella attached only to the ends or poles of the cell. *Planococcus* embraces motile micrococci; *Planosarcina*, motile sarcinæ.

The Naming of Species.—Botanists use two Latin words when speaking of a plant, the first of which denotes the *genus* and the second the *species* to which it belongs: thus the botanical name of red clover is *Trifolium pratense*; of white clover, *Trifolium repens*, both species being different, yet belonging to the same genus. The same obtains when speaking of bacteria: thus we have *Bacillus subtilis* for the hay bacillus, *Bacillus anthracis* for the organism causing anthrax. Unfortunately, this scheme of nomenclature has not been strictly adhered to by many irresponsible workers at bacteriology, but it is hoped that some day the chaos existing in the naming of these organisms will disappear and order take its place. Hundreds of species have been described and named by various writers in different parts of the world, but much comparative work must be

done before it is known what are the differences and relationship of these so-called species and how many are really distinct. There is little doubt that many of them are the same, and a reduction of names must take place in the future. Classification and registration, according to some scheme such as that suggested on p. 60, would facilitate rapid and easy reference and comparison of all bacteria, and students of bacteriological science should adopt a plan of this kind in describing new forms.

6. In addition to the simple types of bacteria already described there is a large family of organisms resembling bacteria in the shape of their individual cells and mode of reproduction, but the cells are enclosed in long, thread-like, tubular sheaths which may be simple or appear branched. Some of these, such as *Cladothrix dichotoma* (p. 128) and species of *Crenothrix*, are not uncommon in impure water and in soil.

7. The following scheme of classification adopted by Migula, although imperfect in some respects, is as satisfactory as any :—

Order I. EUBACTERIA.—Cells colourless, or occasionally containing chlorophyll.

Family I. COCCACEÆ.—Cells globular when free. Division occurs in one, two, or three planes with the formation of chains, plates, or cubical packets. Spores rarely formed.

Genus 1. *Streptococcus.*—Cell-division in one plane, the cells usually remaining attached in the form of a string of pearls. (Fig. 9.)

" 2. *Micrococcus.*—Cell-division in two planes.

Genus 3. *Sarcina.*—Cell-division in three planes; cubical packets produced. (Fig. 9.)

" 4. *Planococcus.*—Motile micrococci.

" 5. *Planosarcina.*—Motile sarcinæ.

Family II. BACTERIACEÆ.—Cells more or less elongated, cylindrical, and straight, without an enclosing sheath. Division at right angles to long axis of cell.

Genus 1. *Bacterium.*—Longer or shorter rods, non-motile. Without endospores.

" 2. *Bacillus.*—Longer or shorter rods, motile. Flagella distributed all over the cell: forms endospores. (Fig. 8.)

" 3. *Pseudomonas.*—Longer or shorter rods, motile. Flagella polar, *i.e.* arranged at ends of the cell. (Fig. 8.)

Family III. SPIRILLACEÆ.—Cells elongated, curved, or spiral, without enclosing sheath. Usually motile.

Genus 1. *Spirosoma.*—Cells rigid, not motile.

" 2. *Microspira.*—Cells rigid, motile usually with one polar flagellum.

" 3. *Spirillum.*—Cells rigid, screw form (Fig. 5), motile with bunch of polar flagella. (Fig. 8.)

Family IV. CHLAMYDOBACTERIACEÆ.—Cells rod-shaped, in chains, surrounded by a common gelatinous sheath. (Fig. 28.)

Several ill-defined genera distinguished, *e.g. Crenothrix*, *Sphærotilus* (*Cladothrix*), and others.

Order II. THIOBACTERIA.—In this group the cells

are colourless, or rose, red, or violet—never green. The cells contain granules of sulphur.

Family I. BEGGIATOACEÆ.—Colourless filamentous forms.

Genus 1. *Thiothrix.*

„ 2. *Beggiatoa.*

Family II. *Rhodobacteriaceæ.*—Cells spherical, rod-shaped, or spiral, containing rose, red, or violet pigment.

CHAPTER III.

GENERAL CONDITIONS WHICH INFLUENCE THE GROWTH OF BACTERIA.

THE growth and multiplication of bacteria are checked or increased by various external conditions, the chief of which are the presence of light, heat, and oxygen, and an adequate supply of water and food-materials; certain chemical substances mentioned later also have a marked influence on the vital activity of these organisms.

1. **The Action of Light.**—Although a small group of bacteria appear to need exposure to light in order to carry on their physiological functions, the majority of them only thrive in very feeble light, or in darkness, and their growth and development are retarded by exposure to diffuse daylight. The action of direct sunlight, even for a short time, results in the weakening of their vital powers, and if continued destroys them altogether. The blue, violet, and ultra-violet rays of light are most destructive to bacteria. The deleterious effect of light appears to be partially dependent upon the presence of atmospheric oxygen, since under anaerobic conditions many bacteria resist sunlight for some time. In certain cases the formation of hydrogen peroxide takes place in the medium in which organisms are growing, and in such instances the death of the bacteria may be attributed to the oxidizing action of this substance.

2. **Heat.**—Like other living organisms, bacteria can only grow and carry on their functions between certain temperatures. The *minimum* or lowest temperature is that below which growth and multiplication ceases, the *maximum* or highest being the point on the thermometric scale above which growth is checked. Between these extremes is the *optimum* or best temperature, at which growth and division is most active; it is usually somewhat nearer the maximum than the minimum point. The minimum for many common species of bacteria lies between 1 to 10° C., the optimum 25 to 35° C., and the maximum between 42 to 50° C. Certain kinds frequently met with in soil, well water, and in the sea, tolerate low temperatures and grow freely at a few degrees above freezing-point. On the other hand, the species *Bacillus thermophilus*, sometimes present in sewage and in the intestinal tract of animals, can develop and multiply at 70° C., a temperature sufficient to kill all animal cells. Many kinds which grow well at 60° C. are also met with in garden soil, manure heaps, and milk, and on the surface of potatoes, straw, hay, and various cereal grains. Although the vital activities of bacteria are reduced or checked altogether by cold, these organisms are not killed even when subjected to temperatures much below the freezing-point of water. Certain forms have, indeed, been immersed in liquid air for some time without showing any loss of vitality. Temperatures of 5 or 10 degrees above the optimum, if continued for a time, act deleteriously on bacteria in a vegetative state of growth; their physiological powers are diminished, and at a few degrees above their maximum death takes place in actively growing cells of most species when they are kept moist. The spores of bacteria, however, resist high

temperatures for a considerable time without losing their germinating power, those of the hay bacillus (*Bs. subtilis*) being uninjured by boiling in water for half an hour or longer. The spores of certain forms isolated from soil have been found to be capable of development after exposure for an hour to a dry heat of 130° C., but a few minutes at 150° C. is sufficient to destroy all kinds of spores.

3. **Water.**—Water is essential for the manifestation and continuance of the phenomena of life in all forms of living beings; without it active life is suspended, and prolonged dryness results in death. The power of resisting drought varies with the kind of organism and with its stage of development; many kinds of bacteria in a vegetative state become weakened or lose their vitality altogether when dried for a few days, while their spores will often withstand drying for many months, or in some cases even for several years.

4. **Oxygen.**—Bacteria differ very much from each other in regard to the need of free oxygen for their growth and maintenance of their vital functions. Some of them are dependent upon a supply of free oxygen in the medium in which they are grown, and are spoken of as *aerobic* species; examples of this class are *Bs. subtilis* and *Bact. fluorescens.* Forming another group are those species which thrive only when free oxygen is excluded, their vital powers being suspended or destroyed by exposure to air for a few hours; these are termed *anaerobic* bacteria, good examples of which are the organisms responsible for butyric acid fermentations. Between these *obligate* aerobes, which soon die unless well supplied with air, and the *obligate* anaerobes, which are checked by the presence of less than .5 per cent. of

the oxygen, there are large numbers of species which are indifferent to the admission or exclusion of air, thriving more or less satisfactorily under both conditions. Those kinds, such as certain forms of lactic acid bacteria, and many of the organisms causing putrefaction, which are usually aerobic, but which can still grow and multiply when the supply of atmospheric oxygen is diminished, are described as *facultative anaerobic* bacteria; they can live as anaerobes when necessity arises. The species which generally live as anaerobes, but are yet capable of existing under conditions which admit of the access of fresh air, are termed *facultative aërobic* bacteria.

5. **Food.**—Food is essential to the life of all forms of living things. Out of it they are able to construct the material used in building up their bodies and in repairing the wear and tear which goes on within them. Moreover, the energy needed to maintain their vital activity is derived chiefly from chemical changes induced in the food which they consume. The substances utilized as food by bacteria are always taken from watery solutions, the dissolved materials diffusing into the interior of the organism through the surrounding cell-walls, although there are no visible openings in the latter. Such passage or diffusion of dissolved substances through membranes which have no apparent openings in them is termed *osmosis*. While a few species of bacteria are known which are able to develop when supplied with very simple chemical compounds, such as carbon dioxide, nitrogen, water, and inorganic salts, the majority of them are unable to grow and multiply unless they are provided with more complex organic compounds, such as sugar, proteins, and similar substances. In respect of the source from which the

organic compounds are derived, two classes of bacteria may be recognized, viz., *saprophytes*, which obtain them from the dead and decaying bodies of plants and animals, or the lifeless organic matter in soil, milk, water, and other common materials; the *parasites*, which feed upon materials present in the tissues of living organisms, their so-called *hosts*, and produce abnormal physiological disturbances or diseases in the latter. Bacteria which produce disease are spoken of as *pathogenic* organisms; examples are the bacilli which cause tuberculosis, anthrax, diphtheria, and enteric fever in man and the lower animals.

The elements composing the food of bacteria are practically the same as those utilised by plants, viz., carbon, hydrogen, oxygen, sulphur, phosphorus, and certain metallic elements, the chief of which are sodium, calcium, magnesium, and iron. The first five enter into the composition of the proteins, which form much of the body substance of bacteria. The metallic elements appear to be necessary for the growth and physiological functions of these organisms, but how they are utilized is not clearly known. It is said that pure cultures of certain species of bacteria have been grown without calcium, sodium, or potassium, but the experiments on which these statements are made are not altogether conclusive; exceedingly minute traces are difficult to eliminate from culture media, and these small amounts are often quite sufficient for the needs of bacteria.

The food elements are obtained from many sources. Some of them, *e.g.* oxygen and nitrogen, may be absorbed in a free state; usually, however, the latter can only be used when presented in a combined form. Potassium, calcium, and magnesium are taken from sulphates,

phosphates, nitrates, and other inorganic salts of these metals. The sulphates and phosphates provide sulphur and phosphorus respectively, and both elements are also derived in part from proteins and other organic compounds.

Carbon can be obtained from a number of substances. The nitrifying organisms are able to procure what they require from the carbon dioxide of the atmosphere, and it is probable that certain sulphur bacteria use this gas as a source of carbon also. Many organic carbon compounds, such as lactic, acetic, tartaric, citric, and other acids or their salts, as well as sugars, alcohols, and their allies, provide carbon; additional sources are proteoses, peptones, and other proteins.

The element nitrogen is obtained by *Clostridium pasteurianum* and species of *Azotobacter* from the free nitrogen of the air, and the nodule organisms associated with the roots of leguminous plants can also make use of free uncombined nitrogen. *Bs. subtilis*, *Bact. fluorescens*, and many other kinds can obtain it from nitrates, especially if the carbon which they need is supplied in the form of sugar. Some species use ammonium compounds, while the majority procure their necessary nitrogen from more complex nitrogenous organic compounds such as the amino-acids and proteins.

For the artificial nutrition of bacteria various liquid media are employed, such as milk, whey, beef-broth, blood-serum, and decoctions prepared from various animal and vegetable substances. Solid jelly-like media are also extensively used; these consist of the liquids just mentioned, or solutions of peptone, sugar, and other nutrient materials, to which is added a certain amount of gelatine or agar-agar. The preparation and use of culture media are described in Chapter IV.

6. **Sterilization.**—Many of the most serious diseases of man and domestic animals are caused and spread by bacteria; these minute organisms are also responsible for the souring of milk and the putrefaction of meat, eggs, and other food materials. It is therefore important to study the means by which their activities can be checked or their vitality destroyed altogether.

Milk, beer, flesh, and all substances from which all living germs have been removed or destroyed, are said to be *sterile*, and will keep indefinitely if, after sterilization, further access of bacteria is prevented.

Various methods of *sterilization* are in daily use, the chief of which are based upon the destructive action of heat, or upon the poisonous nature of certain chemical compounds.

By maintaining a low temperature, food-stuffs may be kept for an indefinite time without change. In a frozen state meat, milk, and fruit of all kinds are sent long distances without damage, and it is well known that fruit, jam, milk, butter, bread, and meat keep best in cool, dry situations. In such cases it must be borne in mind that the low temperature merely checks the development of the bacteria present, and does not destroy them; a sterile condition cannot be produced in this way, and when the temperature is raised the organisms present begin their growth and work of destruction with unabated vigour.

The keeping qualities of foods of all kinds are greatly increased by roasting, baking, boiling, and steaming, for most bacteria are killed by these processes.

The most certain and complete sterility is obtained by the application of heat. A temperature of 150° C., maintained for an hour, is sufficient to destroy all forms of

bacteria, their spores included. Even the boiling of liquids, such as water, milk, and broth at 100° C., kills all living things in them if continued long enough, but certain spores resist this temperature for several hours. Usually it is only needful to heat most liquids to 100° C. for fifteen or twenty minutes to destroy all bacteria in active growth, and a great many of their spores also.

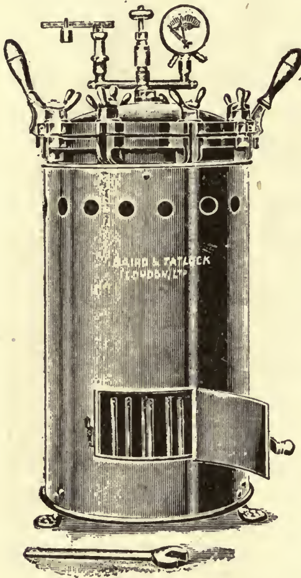


Fig. 13.—Autoclave.

In the case of many liquids it is not possible to heat them in an open vessel above 100° C., for when this temperature is reached steam forms, and evaporation occurs without a further rise in temperature.

If, however, steam is prevented from escaping, the liquid may be heated above 100° C. An apparatus for effecting this is the *autoclave* (Fig. 13). It consists of a strong gun-metal or brass boiler fitted with a lid which can be screwed down. In the latter a safety-valve is provided, as well as a stopcock, by means of which the steam can be allowed to escape when necessary. Attached to the apparatus is a gauge to indicate the pressure of the enclosed steam.

In using the autoclave for the sterilisation of water, milk, or other media used in the cultivation of bacteria, the following instructions may be adopted: Introduce a small quantity of water into the autoclave, and after the

vessels containing the media to be sterilized have been placed within it, screw down the lid. The burners for heating the apparatus should now be lighted. Leave the stopcock open until steam begins to escape freely, then close it, and allow the pressure to rise until the gauge indicates 15 lb., or a temperature of 115° to 125° C. is reached. Lower the gas-burners, and allow the temperature to remain at the figure just mentioned for half an hour; then turn off the gas, and cool the autoclave to below 100° C. before opening the stopcock.

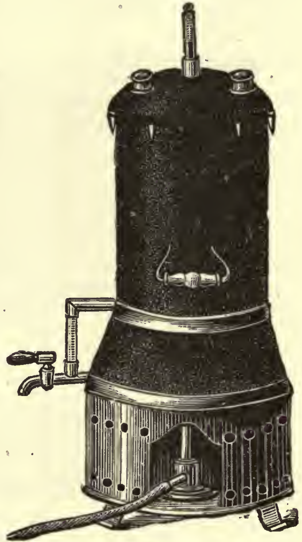


FIG. 14.—Steam sterilizer.

Where the use of the autoclave is prohibited on account of expense or for other reasons, liquids may be rendered sterile by the process of *intermittent sterilization* at a temperature of 100° C. or less. This is effected by heating to 100° C. for twenty minutes on three or four successive days. The first heating kills all bacteria which are in an active growing state, only spores present retaining their vitality. The latter are supposed to germinate in the cool intervals, the organisms arising from them being destroyed by the subsequent heating which they receive; possibly the alternate heating and cooling on successive days may destroy ungerminated spores.

For intermittent sterilization of media a *steam sterilizer* is used (Fig. 14). It consists of an iron or copper cylindrical vessel, covered on the outside with felt, asbestos or other

non-conducting material. On the inside, some little way above the bottom, is a perforated partition upon which the glass flasks, tubes, and other vessels containing the media to be sterilized can rest.

When used water is poured into the sterilizer, and the latter then placed over a Bunsen burner; steam soon forms, and fills the space surrounding the tubes of culture media.

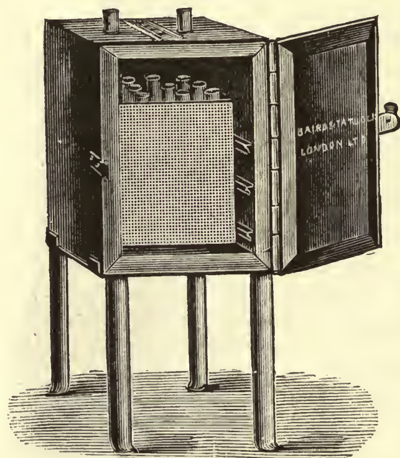


Fig. 15.—Hot-air sterilizer.

An inexpensive substitute for this apparatus is an ordinary large saucepan fitted with a potato steamer.

For the sterilization of glass tubes, flasks, and other laboratory apparatus, which are uninjured by high temperatures, a *hot-air sterilizer* (Fig. 15) is generally used.

This is a cubical sheet-iron box, with a door in front, the whole having double walls, with an air space of about an inch between them.

A temperature of 150° C. or higher is easily maintained in the interior by means of a Bunsen burner placed beneath it; all tubes, flasks, Petri dishes, and similar apparatus should be subjected to this or a higher temperature for not less than half an hour before being used for bacteriological work.

7. **Pasteurization,** or heating for about twenty minutes at 60° to 85° C., followed by rapid cooling to about 10°

C., is a process frequently adopted for the preservation of foods for a limited time. It is largely employed in the dairy industry, and is effective in destroying the sporeless forms of bacteria without materially altering the flavour or composition of the liquids. For its application to milk and cream, see pp. 295-6.

8. **Disinfectants, Antiseptics, and Preservatives.**—The vitality and work of bacteria can be checked or destroyed by various chemical substances. Surgical instruments and premises infected with disease germs are generally sterilized by the application of these compounds. Chemical substances which completely annihilate bacteria are termed *disinfectants*, while those which merely retard the growth of these organisms are known as *antiseptics*. No hard line of separation, however, can be drawn between these two classes, for the effect of any chemical compound depends upon the strength of its solution and the time that it is allowed to act; weak solutions of disinfectants may have an antiseptic action only. Moreover, what would be sufficient to destroy or impede the growth of one species of bacterium will not be equally effective upon another kind.

The following are among the most widely used disinfecting and antiseptic agents:—

Mercuric chloride ($HgCl_2$), in a .1 to .2 per cent. solution, is one of the most certain of disinfectants, but its excessively poisonous quality precludes its general use except in the laboratory under proper control. It is extensively used in surgical practice.

Sulphur dioxide gas (SO_2), or solutions of it in water (sulphurous acid), are effective destroyers of bacteria and fungi. The vapour of burning sulphur,

which consists chiefly of sulphur-dioxide gas, has been employed for a long time for the disinfection of rooms in which fever patients have been nursed. The sulphites of calcium and sodium are also valuable disinfectants, and much used in breweries for the cleansing of foul casks and other utensils.

The so-called "*chloride of lime*" is frequently employed as a disinfecting agent, its effectiveness in this respect depending upon its power of giving off free chlorine gas, which latter very rapidly destroys living bacteria and their spores, especially when moisture is present.

A 10 per cent. solution of *carbolic acid* or *phenol* (C_6H_5OH) has disinfecting properties, but weaker solutions do not effectively destroy the spores of bacteria.

Formaldehyde (CH_2O) during the last few years has been utilized for the destruction of micro-organisms. It is very active, solutions containing .1 to .2 per cent. of the compound being sufficient to kill the spores of most bacteria in less than an hour.

In weak solutions all the above-mentioned substances are good antiseptic agents. To this list of chemicals may be added *thymol*, *toluene*, *chloroform*, *salicylic acid*, *boric acid*, *borax*, *lime-wash*, and *alcohol*, all of which may be employed for retarding putrefaction and other changes induced by bacteria.

One-half to one per cent. of *toluene* ($C_6H_5CH_3$) is frequently utilized to check the development of bacteria in various solutions whose enzyme action it is desirable to study. Chloroform ($CHCl_3$), thymol, and salicylic acid ($C_6H_4OH.CO_2H$) may be used for a similar purpose.

Freshly-burnt quicklime (CaO), when slaked and made into a "whitewash," is a cheap and valuable disinfectant

for application to cattle trucks, the walls and stalls of cowsheds, stables, dairy premises, and other farm buildings, which are liable to become infected with germs of disease, or with bacteria which pollute milk and do damage to dairy products generally.

Soda or *sodium carbonate* (Na_2CO_3) is also useful for the cleansing of domestic utensils of all kinds, its alkaline character having a similar action upon micro-organisms to that of lime.

For the preservation of foods of all kinds from decay and putrefactive changes, the use of poisonous substances, such as mercuric chloride, carbolic acid, and formaldehyde is, of course, prohibited. Much, however, can be done by drying, smoking, heating, and the addition to the food of harmless materials, such as sugar, salt, and saltpetre.

Fruits of many kinds, which often contain 80 to 90 per cent. of water, may be preserved by drying them, the low water-content of the dried product being insufficient to allow of the free growth of bacteria, especially when sugar is present, the latter acting in some degree as an antiseptic agent.

In the case of jam, the addition of sugar is combined with boiling of the material, and a similar practice is adopted in the preparation of condensed milk, 10 or 12 per cent of sugar being added to the milk, the water-content of which has been reduced by evaporation to 25 per cent. or less.

In the "canning" or "tinning" industry, which deals with vast quantities of fish, vegetables, and other perishable foods, reliance is placed upon the destruction of bacteria contained in them by means of heat. The sterilization of the tins and their contents was formerly

carried out in open tanks at a temperature of 100° C., the small opening in the centre of the lid being closed with molten solder after the heating had been sufficiently prolonged to destroy all living germs and their spores.

In order to ensure more certain sterility and reduce the time necessary for the process, the tins are now generally heated in large closed autoclaves under pressure to 115° C. or more.

Common salt ($NaCl$) is one of the most useful substances for the preservation of food. The addition of a small amount assists the keeping quality of butter and cheese; larger quantities are employed in the "curing" of bacon, and a strong brine is capable of preventing the bacterial decomposition of beef, pork, fish, and other kinds of meat.

Boric acid (H_3BO_3) and *borax* ($Na_2B_4O_7 + 10H_2O$) have also an inhibiting effect on the development of bacteria, and have been extensively employed for the temporary preservation of butter, cream, and other dairy products. It is, however, doubtful if these compounds are altogether harmless; if used in sufficient amount to check bacteria, they are liable to produce derangements of the digestive system when the "preserved" food is consumed by human beings.

CHAPTER IV.

THE METHODS OF ISOLATION AND PURE CULTURE OF BACTERIA.

Method of Isolation.—In nature various species of bacteria are met with mixed together, much as we find different kinds of plants distributed in a natural pasture or wood. But just as it is possible to separate from a meadow any single flowering plant and grow it so as to get a large collection of plants all of the same species, so it is possible to isolate and cultivate a single species of bacterium. A collection of bacteria consisting of individuals all alike, *i.e.* belonging to the same species, and free from admixture with any other kinds is termed a *pure culture.* It is only by the study of bacteria in pure cultures that their form, mode of growth, physiological activity, and other features can be determined.

The general principles underlying the separation of bacteria and their pure culture may now be described, but in order to obtain a thorough acquaintance with the work the student must carefully work through the practical exercises appended.

The bacteria to be isolated will perhaps most frequently be distributed in solutions of some sort such as milk or water: where they are present in a solid material, say cheese, butter, or soil, a small portion of the material must be broken up and shaken with pure sterilized water.

In a cubic centimetre or even a drop of the liquid there will usually be hundreds or thousands of bacteria, numbers far too large to be effectually dealt with. A small portion of the milk or other liquid is, therefore, transferred to a large bulk of water, which must, of course, be first rendered free from living bacteria by boiling for some time, and the whole shaken to distribute the organisms evenly. (If the amount taken is a cubic centimetre containing, say, 1000 bacteria, and this is placed in 99 cubic centimetres of water, the diluted mixture will now contain on an average ten of these organisms in every cubic centimetre, a number more easily dealt with.) A cubic centimetre or smaller quantity is now taken from such a mixture and introduced into a test-tube containing 8 to 10 cubic centimetres of sterilized liquid *nutrient medium* or solution of substances upon which bacteria can feed and multiply, to which has been added a small percentage of gelatine, agar-agar, or other material possessing the property of solidifying to a jelly at ordinary temperatures. The nutrient gelatine medium is kept sufficiently warm to render it liquid, and the whole is carefully shaken to separate and distribute the bacteria which have been added to it. The contents of the test-tube are then poured into a glass Petri dish (Fig. 16) or on to the surface of a glass plate: the medium is allowed to cool and solidify, protected from dust. In the thin solid layer of nutrient jelly thus obtained the bacteria are imprisoned, and although they can grow and flourish they cannot move far or become mixed with each other as would have been the case if the nutrient medium had

FIG. 16.—Petri dish.

remained permanently liquid. The dish or plate is placed in an incubator or in a warm room, and after a few hours each individual organism grows and multiplies in the nutrient gelatine, its descendants forming a small aggregation of similar bacteria termed a *colony* (Fig. 17). The colonies produced by the various kinds of bacteria vary considerably in form and colour

FIG. 17.—Common forms of bacterial colonies. 1, Round; 2, lobed; 3, amœboid; 4, mycelioid.

and in other ways. The differences must be carefully studied, as many species are characterized by the kind of colony which they produce on the various media described subsequently.

Ex. 16.—In the practical isolation of bacteria the first step is to clean and sterilize the test-tubes, beakers, and flasks to be used.[1]

(1) **Test-Tubes.**—*New* test-tubes (6 in. by $\frac{5}{8}$ in.) should be cleaned by rubbing the inside with a cotton-wool plug which has been tied with strong thread to the end of a glass rod and dipped in strong nitric acid. After cleaning with acid wash thoroughly with water and allow them to drain until nearly dry, then wash out with strong or absolute alcohol, and when dry put into the mouth of each a cotton-wool plug; place a number

[1] Slides, test-tubes, beakers, stains, and chemicals, as well as incubators and other bacteriological apparatus needed for the practical exercises, can be obtained from Messrs Baird & Tatlock, Cross Street, Hatton Garden, London, E.C.

of the dried plugged tubes in a wire cage and heat in the hot-air sterilizer to 150° C. for one hour (Fig. 15).

(2) **Test-tubes which have been used** and contain nutrient gelatine or other media must be dipped in a saucepan of hot water to liquefy the gelatine. Pour out the contents and then place the tube in a saucepan containing a weak solution of caustic potash, tilting them so that they may be completely filled with the solution without imprisoning air bubbles. Boil for an hour. Take out the tubes and wash them in water several times, clean with nitric acid and alcohol and sterilize as explained above, plugging with cotton-wool as with unused new tubes.

(3) **Flasks, beakers, Petri dishes, pipettes, and other glass apparatus** should be cleaned and sterilized in the hot-air oven in similar manner. All smaller apparatus and pairs of Petri dishes should be wrapped separately in paper before sterilization. They should be stored in a cupboard or drawer away from dust.

Ex. 17.—The next step is the manufacture of a sterilized nutrient medium with addition of gelatine or agar-agar. This requires great care if reliable and consistent results are to be obtained. Bacteria are very sensitive to acids and alkalis; unless the media are suitable in this respect the organisms will not grow. It is found that most bacteria grow satisfactorily when the medium contains 1 per cent. of free normal acid, as explained below, though some commonly met with in the soil require a neutral medium or one which is slightly alkaline. The process of the manufacture of the *nutrient gelatine* may be conveniently divided into four stages.

1. Prepare the nutrient medium (bouillon or broth).
2. Add gelatine.
3. Neutralize carefully and completely, using a solution of phenolphthalein in alcohol as indicator.
4. Add sufficient acid to bring the whole to the proper state of acidity.

Preparation of Standard Nutrient Gelatine.

1. *The broth or nutrient medium.*

The broth is made thus:—Weigh out 5 grams of *Lemco's* extract of meat and 10 grams of fresh Witte's peptone. Dissolve these ingredients in a large flask containing 1000 c.c. of distilled water at ordinary room temperature.

2. *Addition of Gelatine.*

Add to the flask of broth just prepared 100 grams of best sheet gelatine, and allow it to soak for half an hour. Place the flask in hot water in a saucepan and heat slowly until the gelatine and other materials are dissolved, shaking the flask from time to time to mix the contents.

3. *Neutralization process.*

For neutralization the following solutions are required:—

(1) Normal hydrochloric acid.
(2) 1-20th normal acid.
(3) Normal caustic soda.
(4) 1-20th normal caustic soda.
(5) $\frac{1}{2}$ gram of phenolphthalein in 100 c.c. of 50 per cent. alcohol.

Take from the flask 5 c.c. of the nutrient gelatine as prepared above (1 and 2), add it to 45 c.c. of distilled water in a porcelain dish. Boil three minutes to expel any CO_2 and add to it 1 c.c. of the phenolphthalein solution. Run into the hot solution from a burette just sufficient of the 1-20*th normal* caustic soda solution to produce a clear bright pink colour, noting how many cubic centimetres of the soda solution are needed. Repeat again and find the average number of cubic centimetres of soda needed to neutralize 5 c.c. of the nutrient medium. Then calculate how many cubic centimetres will be needed to neutralize the whole of the medium left in the flask. Divide by twenty, this will give the number of cubic centimetres of the *normal* caustic soda solution which will be needed to neutralize the whole of the medium. Add this amount of normal soda to the medium in the flask and test if it is just neutral, as it ought to be, by taking out a few cubic centimetres and adding to it a drop or two of the phenolphthalein. If it is

still acid repeat the above process until the medium is just neutralized.

4. *Standard acidity.*

After neutralization add 10 c.c. of normal hydrochloric acid to every 1000 c.c. of medium. 1.0 per cent. free normal acid is now present, which leaves the medium just sufficiently acid for the growth of most bacteria.

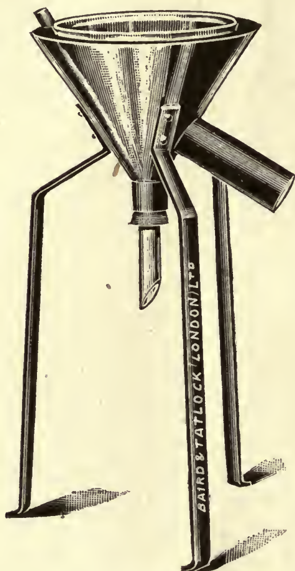


FIG. 18.—Hot-water funnel for filtration of gelatine and agar media.

When working on bacteria common in soil add only 5 c.c. of normal hydrochloric acid instead of 10, as many of these refuse to grow in the more acid medium.

It is usual to indicate these standards of acidity by placing the + sign before the number of cubic centimetres of normal acid used, *e.g.* + 10 and + 5 in the cases mentioned. Where the medium is neutral 0 may be used, and in cases of alkaline media the − sign is employed before the number of cubic centimetres of normal caustic soda or sodium carbonate added to the medium after neutralization.

5. *To clarify.*

Cool the medium to below 40° C. and add slowly the white of an egg well beaten up with a little water; heat in the steam sterilizer or a water bath, and then filter the hot solution through filter paper moistened with boiling water into a sterilized flask: use a hot-water funnel (Fig. 18) when filtering the medium.

6. *Sterilization.*

Take twenty or thirty clean sterilized test-tubes and pour into

each about 8 or 10 c.c. of the medium, taking great care that none of it touches the sides of the tube near the mouth. Plug each tube with cotton wool and heat them all in the steam sterilizer on three successive days for twenty minutes at a time, taking care to have the water boiling before the tubes are placed in the sterilizer. (The medium will keep a long time if stored away from dust, especially if the plugged mouths of the tubes are covered with indiarubber caps.)

Ex. 18.—Pour 250 to 300 c.c. distilled water into a flask, lightly plug the opening with cotton wool, and boil for twenty minutes on three successive days.

When the water is cool put into it 1 c.c. of the liquid containing the bacteria which are to be isolated. Take $\frac{1}{2}$ c.c. or less of the dilute mixture with a sterilized pipette and transfer it to a nutrient gelatine tube, the contents of which have been liquefied by placing the end of the tube in warm water; mix thoroughly, not by shaking but by turning the tube and tilting it so as to avoid bubbles of air in the medium, and then pour it into a sterilized Petri dish. Cool rapidly by placing the Petri dish on a glass plate or on ice for a minute or two; then leave it in a warm room and examine every day for colonies of bacteria. They will appear as spots of varied colour and form on the surface or within the substance of the gelatine medium.

Ex. 19.—Instead of making a dilute solution, as explained above, of the liquid in which the bacteria to be isolated are found, the following plan may adopted. Melt three or four tubes of nutrient gelatine in warm water at 30° to 35° C.; label them 1, 2, 3, and 4 respectively. Take up a drop of the liquid containing the bacteria with a sterile platinum loop and transfer it to the tube labelled 1. Mix carefully and transfer a drop of the mixture to tube 2; from tube 2 transfer a drop to tube 3, and from 3 to 4; mix carefully each time and sterilize the platinum loop each time before use by heating in the flame. Then pour the contents of each tube into four sterilized Petri dishes and leave them in a warm room to incubate.

It will be found that many commonly distributed bacteria have the power of liquefying nutrient gelatine when they begin to grow. When this occurs the colonies soon mix and isolation becomes impossible. Another disadvantage connected with a solid gelatine medium is that, as ordinarily prepared, it melts at 22° to 24° C., and, therefore, cannot be used for growth of organisms at higher temperatures. To avoid these troubles a nutrient medium is used containing agar-agar in place of gelatine.

Agar-agar, or **agar** as it is generally called, is not a protein like gelatine but a carbohydrate substance, which is derived from certain species of Japanese and eastern sea-weeds. It is not liquefied by bacteria, and has the advantage of remaining solid at temperatures up to nearly 80° C.

The substance is sold usually in bars, sheets, or powder, the latter being the most generally used. A small amount (1.5 per cent.) is sufficient when dissolved in water to form a solid moist "jelly" suitable for the growth of many micro-organisms.

Agar is very difficult to dissolve, requiring a temperature of near the boiling point of water to melt it, but when dissolved its watery solution has the remarkable property of remaining liquid and not gelatinizing until the temperature has fallen far below the melting point, namely, to near 39° C.

On account of the sensitive nature of many kinds of bacteria in regard to heat it is important to remember that melted agar media should not be inoculated with bacteria until the temperature has fallen to between 40° to 41° C. On the other hand, the temperature should not be allowed to fall much lower than this, or

solidification may set in and prevent the even distribution of the bacteria in the medium. These precautions should be borne in mind when agar media are used, especially where they are employed for quantitative determination of the number of organisms in solutions.

Preparation of Nutrient Agar Medium.

Ex. 20.—Make a nutrient broth, using—

(1) Lemco's extract of meat, 5 grams.
Witte's peptone, 10 grams.
Water, 500 c.c.

(2) Take 15 grams of agar-agar and scak it for twelve hours in 500 c.c. of water. Mix (1) and (2) and boil or heat in the steam sterilizer twenty to thirty minutes until the agar and other materials are dissolved. Neutralize and bring the hot solution to proper acidity, as in Ex. 17 (3 and 4). Cool to 40°-50° C., and clarify with white of an egg, as in Ex. 17 (5). Then heat in steam sterilizer for 1½ hours, and filter when hot in a steamer or through Chardin filter paper in a hot water funnel, first wetting the filter paper with boiling water. If not clear and free from opaque spots when cool, reheat again, clarify, and pass through a Chardin filter paper. Run 8 to 10 c.c. into test-tubes, and sterilize as in Ex. 17 (6). Agar media filter very slowly. Where an autoclave is available, place the neutralized solution, with the white of an egg added, in the apparatus, and heat for half an hour at two atmospheres pressure or for 45 minutes at 120° C. Turn off the flame, and when cooled to below 100° C. open the autoclave, and the agar medium will be found to filter clear through a Chardin paper in fifteen to twenty minutes.

Ex. 21.—Repeat Ex. 18 and Ex. 19, using the above agar medium instead of nutrient gelatine.

2. **Pure Cultures.**—After the bacteria have been isolated, and the separate colonies allowed to grow on the nutrient gelatine or agar medium, minute portions of each distinctive colony are removed with a fine sterilized

platinum needle and transferred to various liquid media; *e.g.* to broth or milk, in which an abundant pure growth of the particular organism can be obtained. The form and other features of the bacterium can then be studied, as well as its effect on the medium.

In addition to their development in liquids, it is usual to make an examination of the growth of bacteria on the

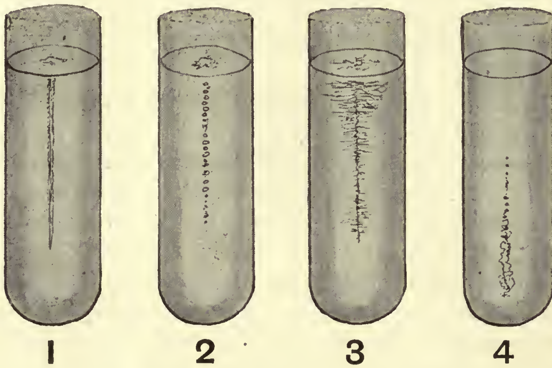


FIG. 19.—Diagram of common forms of stab-cultures.
1, Filiform; 2, beaded; 3, rhizoid or arborescent; 4, beaded stab-culture of anaerobic organism.

surface or deep within the substance of the solid gelatine or agar media. For the latter purpose tubes of solid media are inoculated by means of a platinum needle dipped in a pure culture in broth, or in a colony isolated on gelatine or agar, the needle being pushed deeply into the substance of the solid medium and then withdrawn. The colonies of the bacteria develop along the track of the needle, forming a *stab-culture* (Fig. 19). The needle may, on the other hand, be drawn lightly along the surface of solid media in straight lines, and a *streak-culture* obtained. The medium for the latter purpose may be

melted in its tube and poured out into a sterile Petri dish, where it solidifies into a broad level surface.

Gelatine or agar *slants* may be used for streak cultures instead. These are obtained by melting the medium in its tube, then placing the tube in a sloping direction, and allowing it to remain in this position until the medium cools and solidifies again. In this way a sloped surface is obtained in the tube, on which streak-cultures can be made (Fig. 20).

Although the characters of bacteria as exhibited by

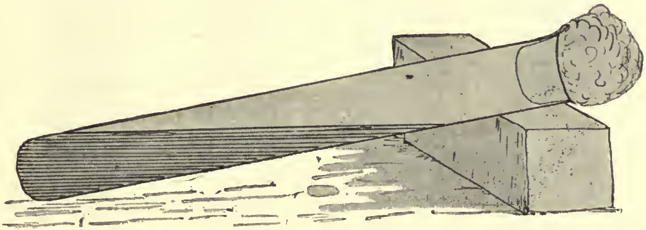


FIG. 20.—Mode of preparing gelatine or agar "slants."

their colonies on solid media are not absolutely constant, it is useful to note points of difference in this respect between the various species.

Colonies are variously coloured, some being white and opaque, others white and translucent, while many kinds are distinctively coloured red, yellow, green, black, and other tints.

The shape of the single colonies may be circular or irregular in outline, with entire or variously indented edges (Fig. 17), and the surface may be flat and smooth, or raised into prominences of various form. All these characters must be carefully observed. In the case of stab-cultures the extent and form of the growth along

and away from the track of the needle must be noted (Fig. 19), as well as the presence or absence of liquefaction of the solid medium (Fig. 21).

Ex. 22.—Make stab-cultures in (1) gelatine and (2) agar tubes of the bacteria which you have isolated.

Incubate and make drawings to illustrate the development of the colonies both on the surface and in the substance of the

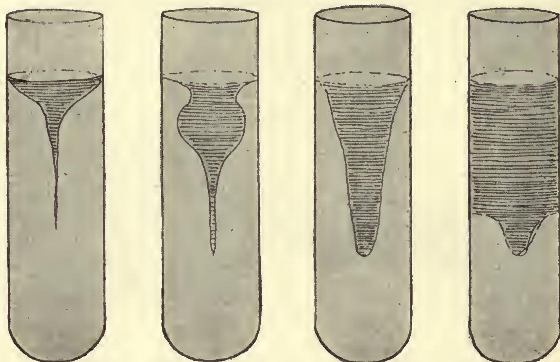


FIG. 21.—Diagram of types of liquefaction following stabs in gelatine media.

media. If liquefaction occurs, note the extent to which it progresses, and the form of space occupied by the liquefied medium.

Ex. 23.—Prepare some gelatine or agar "slants," and make streak-cultures on these. Make drawings of the form of the colonies along the streak, and note any peculiarities of colour, dryness, form of growth, and any other points.

Ex. 24.—Make streak-cultures on solid media in Petri dishes.

3. **Media.**—There is no universal medium in or upon which all kinds of bacteria will grow equally well. Some of the organisms thrive best in beef broth, others in milk or in solutions containing sugar, their requirements in respect of nutrition being very varied. It is, therefore,

necessary to prepare a number of kinds of media before any useful study can be made of the various species of bacteria which are commonly met with.

The way in which the colonies develop in or upon the different media and the form which they assume, as well as the chemical action which the organisms have upon the media, are matters of the greatest importance in assisting us to distinguish one species from another. It is essential that scrupulous care be taken in the preparation of the various kinds of media, and for each kind some definite method should always be adopted and adhered to, since slight modifications in their acidity and composition or in their treatment in the sterilization process often extensively influence the resulting growths of bacteria upon them.

The same species of organism may exhibit many points of difference when grown upon two media, which are very nearly but not quite alike in acidity and composition. The multiplicity of so-called different species of bacteria, and the great difficulty there is in comparing the work and conclusions of those who have carried on investigations in the subject, is due in a measure to the want of uniformity in the media which have been used.

The following are among the most useful media needed by the student. Those required for the study of special organisms present in soil, manure, or dairy products will be mentioned later. In the examination of new or unrecognized species its growth upon a variety of media must be studied and recorded.

In the following exercises instructions are given for the preparation of the kinds of media most frequently needed for the growth and examination of common bacteria.

A stock of each should be prepared for future use.

Ex. 25.—1. Nutrient Beef Broth (Bouillon).—Take 1 lb. of lean beef free from fat, chop it up very fine, with a knife or mincing machine.

Put it in a porcelain dish or glass beaker and pour on it 1 litre of water. Leave it to soak all night in a cool place, covered from dust. Strain the extract through muslin into a saucepan and boil it for one hour. Filter through filter paper into a large flask and make up the volume to 1 litre again. Add to it—

Sodium chloride, 5 grams.
Witte's peptone, 10 grams.

Heat the whole in a steam sterilizer or water-bath for an hour, shaking occasionally until all is dissolved.

Neutralize as in Ex. 17. Heat in steam for quarter of an hour. Neutralize again if necessary and acidify to + 10 as in Ex. 17. Filter into sterile flasks or test-tubes. Sterilize in flasks or in tubes for three successive days for twenty minutes each time.

2. The following varieties of beef broth are occasionally needed :—

Nutrient Beef Broth with Sugars.—Add to the above before final filtration—

(i) Milk sugar or lactose, 20 grams, or
(ii) Grape sugar or glucose, 20 grams.

Ex. 26.—Lemco's Extract Broth (Bouillon).—Dissolve in 1 litre of water at room temperature—

Lemco's extract of meat, 5 grams.
Witte's peptone, 10 grams.

Neutralize as in Ex. 17. Boil for fifteen minutes, make up to 1 litre, neutralize again if needed, and acidify to + 10 as in Ex. 17. Boil, filter into sterile flasks or tubes, and then sterilize on three successive days for twenty minutes each time.

Ex. 27.—Nutrient Gelatine.—Prepare as in Ex. 17, using—

(*a*) Nutrient beef, from Ex. 25, for nutrient medium, or
(*b*) Lemco's extract, as given in Ex. 17 and Ex. 26.

Ex. 28.—The following modifications are frequently needed for special purposes:—

Nutrient Sugar Gelatines.—Add to the ordinary gelatine medium of previous exercise before final filtration—

(*a*) Lactose sugar or lactose, 20 grams, or

(*b*) Glucose sugar or glucose, 20 grams.

Ex. 29.—Litmus-Glucose Gelatine.—Before sterilization add to a neutral gelatine medium 2 per cent. of glucose and enough Kubel-Tiemann's neutral litmus to colour it a purple tint.

Colonies producing acids in this medium become surrounded by a pink halo.

Ex. 30.—1. **Nutrient Agar.**—For its preparation see Ex. 20.

2. **Milk-sugar or Lactose Agar.**—Add to the ingredients mentioned in list (1) Ex. 20 in the preparation of nutrient agar—

Milk-sugar or lactose, 20 grams.

3. **Grape-sugar or Glucose Agar.**—To nutrient agar add—

Glucose, 20 grams.

4. **Glucose-chalk Agar.**—To a neutral agar add enough sterilized precipitated chalk to render the medium white and opaque. When grown on plates of this medium, colonies which produce acids dissolve the chalk and make the medium clear.

5. **Litmus-glucose Agar.**—Before sterilization add to a neutralized agar enough Kubel-Tiemann's litmus to give the medium a purple colour.

Ex. 31.—Milk.—Take some perfectly fresh milk and pass it through a separator.

If the milk is acid, make it just neutral to litmus by adding normal caustic soda solution or sodium carbonate solution.

Place the separated milk in sterilized tubes, and sterilize in steam on three successive days for twenty minutes each time. In order to be quite certain that the milk is free from spores of bacteria, the tubes should be placed in an incubator for twenty-four hours before being inoculated with the bacteria to be studied: if organisms are present curdling or other changes in the milk will usually be noticed.

If separated milk cannot be obtained, take ordinary fresh whole milk; leave it to stand in a beaker in a cool place all night, and in the morning pipette or siphon off the "skim-milk" from below the cream layer.

Ex. 32. — Litmus Milk. — Before tubing the milk, add enough Kubel-Tiemann's neutral litmus to tinge it a purple colour; then sterilize as advised in previous exercise.

When inoculated with an acid-forming organism, the purple colour becomes changed to pink.

Ex. 33.—Whey as Medium.—Take a litre of separated or skim-milk in a dish or bowl, and pour into it 10 c.c. of distilled water, to which 8 to 10 drops of commercial rennet extract have been added. Stir briskly for a few seconds, so as to mix the whole and allow the milk to coagulate. When the curd is formed, cut it with a knife and leave it for an hour so that the whey may separate. Then filter the whey through muslin. Add the white of an egg whipped up with 100 c.c. of water. Heat in steam sterilizer for an hour. Filter into test tubes and sterilize in the usual way.

Ex. 34.—Potato.—Procure some long sound tubers. Wash them in water, and scrub off all soil with a nail brush. Cut off the two ends, and with a cork-borer cut cylinders from the potatoes. Divide the cylinders diagonally with a sterile knife, and wash the pieces twelve hours in running water, or wash them in several changes of water until no milkiness is observed.

Place each of the washed halves, thick end downwards, in sterilized Buchner or potato tubes (Fig. 25), the bulbs of which are half-filled with distilled water. The tubes can be tilted so that the water touches the bases of the slices and keeps them moist when bacteria are being cultivated upon them. If no special tubes are at hand, place a piece of wet cotton wool at the bottom of ordinary wide test-tubes before putting in the potato slices.

Sterilize on three successive days in steam for thirty minutes each time. It is very necessary to observe great cleanliness in preparing the potato slices, as the soil upon the tubers frequently

contains spores of bacteria which are highly resistant to heat and not readily killed by steaming.

4. **Identification, Classification, and Registration of Bacteria.**—In determining the specific character of a bacterium, its morphological and physiological features must be thoroughly examined, and in describing a new organism these features must be exhaustively studied and the results accurately recorded in order to facilitate future comparison and reference. No description should be considered complete which does not contain full information respecting the following points :—

(*a*) The form, size, and motility of the organism.

(*b*) The presence or absence of spore formation ; the shape, size, and position of the spores in the parent cells.

(*c*) Staining quality by Gram's and other methods.

(*d*) The form and colour of the surface and deep colonies in gelatine and agar media. Is the gelatine liquefied ?

(*e*) The aerobic or anaerobic character and the growth and behaviour at low and high temperatures (20° and 37° C. respectively).

(*f*) Growth on potato.

(*g*) Action upon milk : coagulation and acid formation.

(*h*) Its action in broths containing various sugars ; the power of forming acids and gas in these media. Its relative growth in broth with + 10, neutral and − 10 reactions.

(*i*) The power of reducing nitrates with the production of gas.

The practical testing of the various characters of the organisms is dealt with in subsequent exercises.

A simple and convenient method of classifying and registering the character of bacteria which is especially

useful in the case of nearly allied forms is that adopted by the Society of American Bacteriologists. It is an adaptation of the Dewey system of classifying books—a record of the characteristic features of the various organisms being kept in accordance with the numerical system given below.

The registration of organisms in this manner would greatly facilitate the comparison of most forms met with in the course of research with those already known, and it is hoped that some plan of this kind will be adopted by all workers.

Numerical System of recording the chief Characters of a Bacterium.

Group number.	
100.	Endospores produced.
200.	„ not produced.
10.	Aerobic.
20.	Facultative anaerobic.
30.	Strictly anaerobic.
1.	Gelatine liquefied.
2.	„ not liquefied.
.1	Acid and gas from glucose.
.2	„ and no gas from „
.3	No acid from glucose.
.4	No growth with „
.01	Acid and gas from lactose.
.02	„ and no gas from „
.03	No acid from lactose.
.04	No growth with „
.001	Acid and gas from saccharose.
.002	„ and no gas from „
.003	No acid from „
.004	No growth with „

Group number.

.0001	Nitrates reduced with evolution of gas.
.0002	„ not reduced.
.0003	„ reduced without gas production.
.00001	Fluorescent.
.00002	Violet chromogen or colouring matter.
.00003	Blue „
.00004	Green „
.00005	Yellow „
.00006	Orange „
.00007	Red „
.00008	Brown „
.00009	Pink „
.00000	Non-chromogenic.

The genus is indicated by a symbol or contracted term preceding the number, *e.g.*

Bacterium coli, Esch., is recorded thus :—*Bact.* 222.11110
Streptococcus lacticus, Kr., „ „ *Str.* 222.22220

Other features not included in the group numbers may be dealt with in tabular form as shown below, the presence or absence of a feature being indicated by a plus (+) or minus (–) sign. For example, the characters of

				Gelatine plate.				Milk.					
		Gram's stain.	Growth at 37° C.	Liquefaction.	Round.	Lobed.	Rhizoid.	Acid.	Alkaline.	Curdled.	Digested.	Indol.	Vosges and Proskauer.
Str. lacticus, Kr., are :—	*Str.* 222.22220	+	+	–	+	–	–	+	–	+	–	–	–
Bact. acidi lactici, Hüppe,	*Bact.* 222.11000	±	+	–	+	–	–	+	–	+	–	+	–

The growth upon agar and potato or in broth and other media can also be given in a small space by judicious selection of terms for such a table.

Ex. 35.—Determine if the organisms forming the colonies on any of the agar or gelatine plates which you have prepared are motile or non-motile.

To do this, take a hollow-ground "*hanging drop*" slide and sterilize it by passing it four or five times through the Bunsen flame. Now smear a thin ring of vaseline round the edge of the hollow in the slide.

Then take a cover-glass and, after passing it through the flame two or three times, transfer a drop of sterile broth to the centre with a platinum loop, taking care that the drop does not flow to the margin of the glass. Now introduce a small portion of the culture to be tested into the drop with a platinum needle. Lift the cover-glass with forceps and turn it over very rapidly so that the drop is hanging on the under surface; then lower it on to the ring of vaseline. The drop should now be hanging in the enclosed space without touching the slide, as in Fig. 22. As communication between the hollow space and the outside air is prevented by the vaseline, the drop does not readily dry up.

FIG. 22.—Section of hanging-drop slide.

Keep the whole at room temperature or 22° C., and examine with the microscope every half hour for two or three hours. If the organisms are motile their movement will be readily perceived, especially if a small diaphragm opening is used.

Care should be taken to avoid bringing the objective down on the cover-glass: if difficulty arises in focussing, make a small dot on the surface with ink and focus this first.

Ex. 36.—Test some of the organisms which you have isolated for gas production—

(*a*) **With Durham's Tubes.**

First add about 10 c.c. of nutrient beef broth containing glucose (see 2, Ex. 25) to several Durham's fermentation tubes

(Fig. 23); put in the small tubes open end downwards; plug with cotton wool and sterilize for half an hour on three successive days: the small inner tube in each becomes completely filled with the broth.

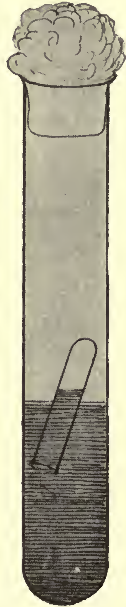


Fig. 23.—Durham's fermentation tube.

Now inoculate the medium with small portions of the different colonies to be tested and incubate at 37° C. If fermentation occurs the small tube becomes partially filled with gas.

(*b*) **With Saccharimeter Tubes.**

The closed end of the tube should be filled *completely* and half the bulb also with glucose beef broth (Fig. 24, *a*). After sterilization, inoculate and incubate at 37° C.

If gas is produced some of it will collect near the closed end of the tube (Fig. 24, *b*).

It is useful to have the limb of some of the tubes graduated. Note the amount collected after two or three days; then add 1 or 2 c.c. of strong caustic potash solution.

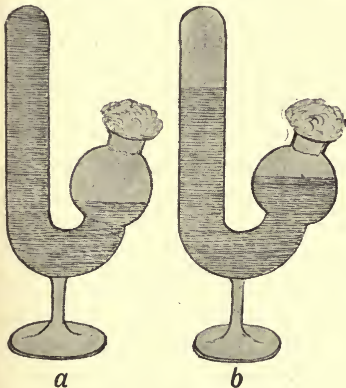


Fig. 24.—Saccharimeter tubes.
a Full; *b* after inoculation and production of gas.

Some of the gas is absorbed, the diminution representing the amount of carbon dioxide present; what is left is in most cases hydrogen, so that the ratio of CO_2 to H can be calculated.

If the medium is cloudy only in the bulb, the organism is aerobic; where the upper end of the tube is cloudy and the medium below remains

clear, the organism is anaerobic; facultative anaerobes usually render the whole contents of the tube turbid.

Inoculate a specimen of both the above tubes of media with *Bact. coli* and note the results.

Ex. 37.—Melt a few tubes of nutrient beef broth gelatine containing glucose (see Ex. 25), and inoculate them with various organisms to be tested for gas production.

Allow them to cool and solidify, then incubate at 20° C.; if gas is produced it collects in bubbles throughout the medium.

Try a tube with *Bact. coli.* and note the results.

Cultivation of Anaerobic Bacteria.—In the cultivation of anaerobes it is necessary to devise some means for excluding the oxygen from, or for removing it from, the space surrounding the medium.

The air may be expelled from the medium by boiling; it may be extracted with the air-pump, or it may be displaced by hydrogen, nitrogen, or some other gas.

The simplest arrangements for the growth of anaerobes are the following:—

(1) Cultivation in a deep stab in agar or gelatine.
(2) Cultivation in a Buchner's tube.

Ex. 38.—(1) **Anaerobic Cultivation in a Deep Stab.**—Take a tube which is more than half full of agar or gelatine medium and place it in a beaker of warm water; heat to boiling for five minutes so as to expel the dissolved air from the medium.

Cool the tube in water, and when the agar or gelatine is solidified, make a deep stab to near the bottom of the tube with a long platinum needle, upon which are the organisms to be grown; rotate the needle and then withdraw it. In the recently melted medium the track of the needle closes up.

Now gently melt the upper layer of the medium by rotating it rapidly near a Bunsen flame; this more effectually closes the track.

Another plan is to pour into the tube two or three centimetres of melted agar at a temperature not higher than 40° C.

After inoculation plug the tube and cover the opening with a tight-fitting rubber cap washed in a dilute solution of mercuric chloride.

Or the tube may be placed in a Buchner tube, described below, without the rubber cap.

Under these conditions most anaerobic organisms readily form colonies in the lower part of the medium.

(2) **Growth of Anaerobes in a Buchner Tube.**—This is the most generally adopted method.

The Buchner tube consists of a strong wide-bored test-tube, near the base of which is a constriction (Fig. 25).

Make a streak of the organism to be grown on an agar or gelatine slant or potato-slice.

Then take 1 to 2 grams of pyrogallic acid and dissolve it in 2 to 3 c.c. of water; to this add 10 to 15 c.c. of a 20 per cent. solution of caustic potash.

Pour the mixture into the Buchner tube and also place the test-tube containing the bacteria inside it as soon as possible.

Plug the opening of the Buchner tube immediately with a close-fitting rubber stopper, slightly greased with vaseline: to make the joint gas tight it is advisable to cover it with melted paraffin wax.

FIG. 25.—Buchner tube, arranged for cultivation of anaerobic organisms.

The mixture of pyrogallic acid and caustic potash absorbs the oxygen in the tube, leaving only the nitrogen of the air with a small trace of carbon monoxide which is produced from the mixture. In this atmosphere most anaerobic organisms grow satisfactorily.

(3) **In Yeast Flask in Hydrogen or other Inert Gas.**—

Anaerobic organisms when grown in broth, milk and other liquid media, very frequently give rise to the evolution of much gas, which, in a completely sealed vessel, is likely to lead to dangerous explosions.

On this account it is best to arrange for their culture in liquid media in yeast flasks, arranged as indicated in Ex. 140, where a simple mercury valve allows of the escape of gas without permitting the entrance of air.

CHAPTER V.

FERMENTATIONS AND THE ACTION OF ENZYMES.

1. WHEN barley grains are supplied with an adequate amount of water and kept warm they begin to germinate. The embryo plant within develops and its root and stem break their way out of the grain. At the same time the starch of the floury part or endosperm is changed into sugar, the sweet taste of which can be recognized in the sprouted grain. The change of the starch into sugar is brought about by the action of a peculiar nitrogenous compound secreted by the cells of the embryo plant. This substance, known as *diastase* is an example of an extensive class of bodies termed *ferments* or *enzymes*, which are produced in various organs of living plants and animals. It is found in all germinating seeds, tubers, and other parts of plants which contain starch, and serves the purpose of changing this solid reserve-product into soluble sugar and a gum-like substance, dextrin, which are able to diffuse through the body of the plant to wherever nutrition and growth are going on.

2. A great many kinds of enzymes are known: some of them have a specific action on certain kinds of sugars, others are concerned with chemical changes in proteins, urea, cellulose, and many other organic compounds. While some of them, such as pepsin and trypsin men-

tioned later, can act upon several different kinds of compounds of the same class, many examples are known of enzymes which are very specially concerned with the decomposition of one definite kind of chemical substance only and are inactive upon any others even of very similar or allied composition. They are generally soluble in water, and although their solutions may be obtained in a concentrated form by extracting the tissues of plants and animals containing them, they have not yet been isolated in a pure state. One of their most striking properties is the power which very small quantities have of decomposing or changing large amounts of the particular chemical compounds upon which they work, and that without suffering much diminution or loss during the process. Enzymes are active only within a limited range of temperature. Those obtained from the tissues of warm-blooded animals carry on their work best at about 37° C., while those of vegetable origin are generally most effective about 25° C. At low temperatures their action is checked but not permanently destroyed; they are, however, destroyed when their solutions are heated to 70° or 80° C. for a short time. In a dry state they resist a temperature of 120° C. or more without damage. The majority act only in neutral or faintly acid solutions, but a few are effective in an alkaline medium.

As already indicated enzymes are very sensitive compounds and much influenced by external conditions. They are readily destroyed by high temperatures and strong acids or alkalis, but neutral salts are less injurious and often precipitate the enzymes along with proteins and other substances with which they are associated in living tissues of plants and animals.

Sunlight only affects them to a slight extent. Many poisons, such as chloroform, phenol, thymol, salicylic acid, sodium fluoride, and alcohol, which check the vital activity of organisms or destroy them altogether, have comparatively little effect on enzymes.

By adding one or other of these substances in small amounts to liquid cultures of bacteria it is possible to destroy or check the latter without materially affecting any enzymes they may have formed and set free in the medium.

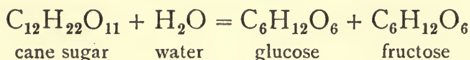
3. When fruit juice or a solution containing sugar is exposed to the air in summer it very frequently loses its sweet taste and small bubbles of gas arise within it. The juice becomes frothy, and a chemical examination shows that the sugar is decomposed with the formation of alcohol and carbon dioxide gas, the latter escaping in small bubbles and producing an effect resembling the boiling of water. The peculiar chemical change is found to be due to the presence in the liquid of vast numbers of living yeast-cells, which have grown and multiplied from a few which have fallen in from the air or which were adhering to the fruit when the juice was first prepared. The term *fermentation* (from *fervere*, to boil) has been applied for a long time to decompositions like this in which the frothing of the liquid is a characteristic phenomenon; it is, however, now extended to include chemical changes brought about by enzymes such as the diastase, even where no gas is produced, so that its original meaning has disappeared.

The living yeast plant is sometimes spoken of as an *organized* ferment in contradiction to the non-living *unorganized* ferments or enzymes, of which diastase previously mentioned is an example. All early attempts to

extract an enzyme or lifeless compound from yeast capable of decomposing sugar into alcohol and carbon-dioxide failed, and the alcoholic fermentation was assumed to be the result of the activity of living yeast cells only. About 1896, however, Buchner ground up yeast with very fine sandy material in order to rupture the cells, and then subjected the dough-like mass to heavy pressure. By this means he extracted a juice which, in the complete absence of living cells, decomposed small amounts of sugar into alcohol and carbon-dioxide: the juice was found to contain an enzyme which he named *zymase.* This result brings the alcoholic fermentation by yeast into line with the changes produced by unorganized ferments or enzymes. More recently, lifeless products have also been obtained from lactic bacteria which are able to produce lactic acid from sugars, and, similarly, enzymes have been obtained from the acetic bacteria which are able to oxidize alcohol to acetic acid just as the living organisms do, except to a smaller extent. The view, therefore, is gradually gaining ground that fermentations of all kinds, whether associated with living cells or not, are due to structureless non-living enzymes, and that it is perhaps better to speak of *intra-cellular* or *endo-enzymes* and *extra-cellular* ferments or *ecto-enzymes*, the former term being applied to enzymes which ordinarily carry on their work within the cells in which they are produced, while the latter is reserved for those which are excreted and are able to act independently of the cell. It must, however, be noted that there are still many fermentations which do not take place except in the presence of living organisms; with many of these we shall be specially concerned later.

4. It will be useful here to notice briefly some of the

most important types of fermentation and the different varieties of enzyme action ; those of agricultural interest will be dealt with in greater detail in subsequent chapters. Enzymes usually change or decompose the substances upon which they act, forming from them bodies of simpler constitution. The chemical reactions in the majority of cases are examples of *hydrolysis*, a form of decomposition or dissociation which involves the fixation of the elements of water. One molecule of a substance is split into two with the addition of water (H_2O): for example, under the influence of the enzyme invertase, which is secreted by yeast, cane sugar is split into two simpler kinds of sugar, according to the following equation :—



$$\underset{\text{cane sugar}}{C_{12}H_{22}O_{11}} + \underset{\text{water}}{H_2O} = \underset{\text{glucose}}{C_6H_{12}O_6} + \underset{\text{fructose}}{C_6H_{12}O_6}$$

A small number of ferments are known which affect the *oxidation* of compounds ; examples of this type are the vinegar plant and *Bacterium aceti*, which convert alcohol into acetic acid. Some few ferments function as *reducing agents*. The fibrin ferment of blood and rennin in the stomach of animals bring about the *coagulation* or "clotting" of proteins in blood and milk respectively : these are termed "*clotting*" *enzymes*.

In addition to those which effect simple hydrolysis, oxidation or reduction, a large number of ferments are known which induce chemical reactions of a much more complicated character. Many obscure processes, such as those of putrefaction and the ripening of cheese, are due to the activity of ferments, but the various stages of the transformations which occur are imperfectly understood.

In naming the enzymes it is usual to add the suffix *-ase* to the root of the name of the substance upon which the

ferments were first observed to act; for example, Duclaux named one of the enzymes which attacks the casein of cheese, *casease*; that affecting maltose sugar is termed *maltase*, the ferment which changes amylum or starch is an *amylase*, the urea ferment *urease*, and so on. Similarly the proteid-destroying class are described as *proteases*. This nomenclature, however, is not strictly adhered to, since it is customary to retain the names trypsin and pepsin for two common proteases, and the terms emulsin, ptyalin are still in ordinary use.

From many points of view it is convenient to classify the enzymes according to the nature of the substance which they are able to ferment. The most important organic compounds which undergo fermentation are the carbohydrates, proteins and fats. These form the greater part of the body substance of all animals and plants, and enter into the composition of food stuffs used by many kinds of living organisms. It will be useful to refer briefly here to the general character of the three classes, and give a short account of the more commonly occurring representatives in each.

1. **Carbohydrates.**—To this class belong cellulose, starch, various kinds of sugar, and many other widely distributed natural products. They all contain carbon, hydrogen and oxygen, the two latter elements being present in the same proportion as they exist in water: hence the name hydrate of carbon or carbohydrate. They may be divided into three groups:—

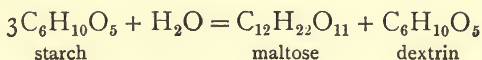
(i) The polysaccharoses, amyloses or starch group.

(ii) The disaccharoses, sometimes known as the saccharose or cane-sugar group.

(iii) The monosaccharoses, termed glucoses or the grape-sugar group.

(1) **Polysaccharoses.** (*a*) **Starch.**—The chief Polysaccharose is *starch*, a carbohydrate having the empirical formula, $C_6H_{10}O_5$, but really possessing a much higher molecular weight. It is a reserve food very commonly distributed in the cells of tubers, roots, seeds, and other parts of plants, and occurs in the form of solid grains built up of a layer of material arranged round a more or less central nucleus or hilum. The substance of the grain consists of starch or amylose, a compound which is insoluble in cold water but swells up and partially dissolves in hot water, forming "starch paste." A solution of iodine in potassium iodide turns it a characteristic deep blue colour. When boiled for some time with dilute acids it is finally converted into grape-sugar, intermediate products, such as maltose and gummy substances being first produced.

The Hydrolysis of Starch by Ferments.—When thin starch-paste (prepared by boiling about a gram of starch in 50 c.c. of water) is mixed with an extract of malt and kept warm, the liquid gradually loses its opalescent appearance and finally becomes clear. If small portions of the mixture are removed and tested at short intervals, with a solution of iodine and potassium iodide, the first portion containing unaltered starch is coloured blue, the second purple, later a reddish brown colour is observed, and finally the solution when tested remains colourless. The changes going on in the starch-paste are due to the action of the enzyme diastase (amylase) contained in the malt extract. At a temperature of 50° to 60° the starch is hydrolysed into maltose and dextrin, a gum-like substance.



$$\underset{\text{starch}}{3C_6H_{10}O_5} + H_2O = \underset{\text{maltose}}{C_{12}H_{22}O_{11}} + \underset{\text{dextrin}}{C_6H_{10}O_5}$$

About 81 per cent. of maltose and 19 per cent. of dextrin are found when the reaction is at an end. Under certain conditions diastase will convert the dextrin into maltose, so that the complete and final hydrolysis of starch by this enzyme results in the formation of maltose only. In all cases, however, a number of intermediate dextrins are first produced, and it is to the presence of these that the colour changes, previously mentioned, are due, the purple colour being given by starch and a certain amount of one or more kinds of dextrin, the reddish brown tint by the latter in the absence of starch, the final products, namely, maltose and dextrin, being uncoloured by iodine.

Many pathogenic bacteria are able to produce amylolytic or starch-splitting ferments; *Bs. subtilis, Bs. megatherium*, *Clostridium butyricum*, and other butyric bacteria, as well as species of *Proteus* and bacteria isolated from cereal grains and soil, have been found to yield enzymes of this class also.

Ex. 39.—Cut thin sections from a piece of potato tuber and from a wheat grain: examine with a low power and make drawings of the starch-grains within the cells observed.

Ex. 40.—Make a strong solution of potassium iodide in water and add to it a few crystals of iodine. Allow the mixture to stand for twelve hours, and shake occasionally in order to facilitate the solution of the iodine. When the latter is all dissolved, add more water until the whole is the colour of dark sherry.

When examining the starch-grains place a drop of this solution near the edge of the cover-slip so that it may run under the latter and come in contact with the starch-grains. Note the change in colour of the starch-grains.

Ex. 41.—Make an extract of malt diastase as follows: Shake

up five grains of ground malt with 50 c.c. of cold water, and after allowing it to stand for four hours, filter so as to get a clear solution.

Next grind some starch with water in a mortar and pour a little of the mixture into a 200 c.c. flask of boiling water. When cool pour about 20 c.c. of this thin starch paste into three test-tubes: show the presence of starch by adding a few drops of the solution of iodine mentioned in Ex. 40 to one tube, and to the other two tubes add 3 or 4 c.c. of the diastase extract, and warm them to 60° C. Test for the presence of starch in one of these two tubes by taking out at intervals of five minutes a few drops with a pipette and adding them to weak solutions of iodine kept in a series of test-tubes.

After a time the starch is changed into sugar and dextrin: When this has happened show the presence of the sugar by means of Fehling's solution (Ex. 44).

See if Fehling's solution is acted upon by the thin starch paste when no diastase is added.

(*b*) **Cellulose.**—Somewhat allied to starch is *cellulose* and its derivatives, which form the solid cell walls of plant tissues: they are produced by the activity of the protoplasm or living material within the cells. What may be regarded as typical cellulose may be obtained from cotton wool, paper, and flax fibre. It has an empirical formula near $C_6H_{10}O_5$, is insoluble in water, dilute acids or alkalis, but dissolves in ammoniacal cupric oxide and other solvents. Cellulose usually stains blue when treated with strong sulphuric acid and iodine. Several complicated compound celluloses, such as the ligno-celluloses of timber and the adipo-celluloses of cork, are known, but these cannot be mentioned further here.

Hydrolysis and Fermentation of Cellulose.—The cellulose walls of the endosperm of many seeds are hydrolysed

apparently with the formation of sugars by an enzyme named *cytase*, and a cellulose-dissolving enzyme is possessed by many fungi, both parasitic and saprophytic, which enables them to penetrate the epidermis, wood and other tissues of plants.

In a later chapter (Chap. XIV.) is discussed the fermentative decomposition of the cellulose of leaves, stems, straw, paper, and similar materials by means of certain species of bacteria present in manure heaps, the mud of swamps, and the intestinal tract of certain herbivorous animals.

(ii) **Disaccharoses.** — The disaccharoses have the formula $C_{12}H_{22}O_{11}$. Three of the best known representatives are cane-sugar, milk-sugar, and malt-sugar.

(*a*) *Cane-sugar or Saccharose* is a very sweet crystalline carhohydrate found in abundance in the cell sap of the sugar cane, beetroot, and other plants.

When boiled with dilute acids it is transformed into grape-sugar and fruit-sugar, the mixture of these two being sometimes spoken of as *invert-sugar.*

An inverting enzyme, *invertase,* which effects a similar chemical change in cane-sugar, is widely distributed in nature: it is met with in yeast, and is possessed by *Bs. megatherium*, *Bs. vulgatus,* and other bacteria.

Cane-sugar does not reduce Fehling's solution, and cannot be fermented directly by yeast.

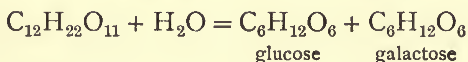
Ex. 42.—Dissolve some ordinary sugar in water and

(i) Test some of the solution for a "reducing" sugar as in Ex. 44.

(ii) Take 10 c.c. of the solution and add to it three or four drops of strong hydrochloric acid: boil for twenty minutes, and after neutralizing the acid with a solution of sodium carbonate, boil and test again with Fehling's solution.

(*b*) *Milk Sugar or Lactose*, $C_{12}H_{22}O_{11} + H_2O$, is found only in the animal kingdom, being an important constituent of the milk of various animals. It occurs in whey obtained in cheesemaking, and is a crystalline compound with only a faint sweet taste. Milk-sugar is not so soluble in water as cane-sugar: it reduces Fehling's solution.

When treated with hot dilute acid it suffers hydrolysis, and splits up into glucose and galactose thus:—



$$C_{12}H_{22}O_{11} + H_2O = \underset{\text{glucose}}{C_6H_{12}O_6} + \underset{\text{galactose}}{C_6H_{12}O_6}$$

The enzyme capable of effecting this change is termed *lactase.* It is apparently possessed by many kinds of bacteria.

In the lactic fermentation it is probable that the first action of the bacteria is a hydrolytic process, the two sugars produced being subsequently converted into lactic acid: the chemical changes in lactic fermentation, and the bacteria to which they are due, are discussed at greater length in Chap. XVII.

Ex. 43.—Dissolve some milk-sugar in water and
(i) Test its reducing power with Fehling's solution (Ex. 44).
(ii) Taste some of the sugar.

(*c*) *Malt-Sugar or Maltose* is a compound with the same empirical formula, $C_{12}H_{22}O_{11}$, as the two preceding sugars, and is produced by the action of diastase and other enzymes upon starch. Hot dilute sulphuric acid changes it into grape-sugar. Yeast is able to ferment it, with the production of alcohol and carbon dioxide.

(iii) **Monosaccharoses.**—The most commonly occurring monosaccharoses are *glucose*, or grape-sugar (formerly

termed dextrose), and *fructose*, or fruit-sugar (sometimes called lævulose). Both have the empirical formula, $C_6H_{12}O_6$. A mixture of these two sugars are formed when the cane-sugar is "inverted" by boiling with acids, or by the action of certain enzymes.

(*a*) *Glucose or Grape-sugar* is found in company with an equal amount of fructose in most ripe fruits, and can be produced by the hydrolysis of starch, cellulose, and other polysaccharoses, the change being effected by boiling with dilute sulphuric acid. It is not very sweet.

(*b*) *Fructose or Fruit-sugar* is met with in fruits along with grape-sugar, as stated above, and in "invert-sugar."

(*c*) *Galactose* is another monosaccharose of the same empirical composition as the two preceding compounds, and is prepared by boiling milk-sugar or certain gums with dilute sulphuric acid.

These sugars reduce Fehling's solution, and are directly fermentable by yeast and certain species of bacteria.

Ex. 44.—Dissolve 35 grams of copper sulphate in 500 c.c. of water, label this solution A: then dissolve 160 grams of caustic potash and 173 grams of sodium potassium tartrate in 500 c.c. of water and label the solution B. By mixing equal quantities of A and B, Fehling's solution is produced. (The solution A and B should be kept separate and only mixed when needed, as the mixture does not keep long.)

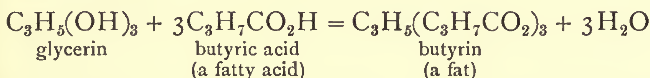
Squeeze a few drops of grape juice into a test-tube containing 10 c.c. of the Fehling's solution: heat over a Bunsen flame and note the reddish precipitate of cuprous oxide (Cu_2O).

Test the juice of ripe plums and other fruits in the same way.

2. **Fats and Oils: Lipase.**—Fats and oils contain the same elements as carbohydrates, namely, carbon, hydrogen, and oxygen, but the oxygen is in smaller proportion to the hydrogen than in the latter compound.

They are abundant in many animal and vegetable tissues, generally in the form of round liquid globules. They are present in stems and leaves, in flax seeds and the seeds of rape, cotton, and other plants, where they are reserves of food for use in the early nutrition of the seedling. Milk contains a considerable amount of fat; it is also found stored up in the animal body, and is reabsorbed into the blood when occasion requires, its oxidation assisting in the maintenance of the body temperature.

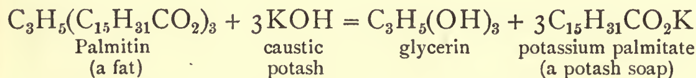
Fats and oils, as usually found in nature, are not single pure substances, but mixtures of several chemical compounds termed *glycerides*, which are formed by the union of glycerin with a fatty acid; *e.g.*—



$$\underset{\text{glycerin}}{C_3H_5(OH)_3} + \underset{\substack{\text{butyric acid}\\\text{(a fatty acid)}}}{3C_3H_7CO_2H} = \underset{\substack{\text{butyrin}\\\text{(a fat)}}}{C_3H_5(C_3H_7CO_2)_3} + 3H_2O$$

These compounds are chemically analogous to salts, and are spoken of as ethereal salts of glycerin, the glycerin in them playing the part of a base.

When treated with caustic soda or potash, the alkalis turn out or set free the glycerin, and unite with the fatty acid to form *soaps*, which are potassium or sodium salts of the fatty acids; *e.g.*—

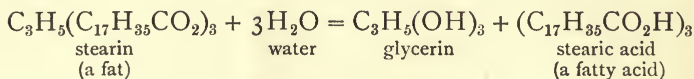


$$\underset{\substack{\text{Palmitin}\\\text{(a fat)}}}{C_3H_5(C_{15}H_{31}CO_2)_3} + \underset{\substack{\text{caustic}\\\text{potash}}}{3KOH} = \underset{\text{glycerin}}{C_3H_5(OH)_3} + \underset{\substack{\text{potassium palmitate}\\\text{(a potash soap)}}}{3C_{15}H_{31}CO_2K}$$

Such chemical action is termed *saponification.*

In many seeds and other parts of plants, as well as in the secretions of the pancreas in animals, an enzyme named *lipase* occurs, which has the power of decomposing fats; the change is a hydrolytic process, the fats

being split up into glycerin and the corresponding free fatty acid, thus :—



$$\underset{\substack{\text{stearin} \\ \text{(a fat)}}}{C_3H_5(C_{17}H_{35}CO_2)_3} + \underset{\text{water}}{3H_2O} = \underset{\text{glycerin}}{C_3H_5(OH)_3} + \underset{\substack{\text{stearic acid} \\ \text{(a fatty acid)}}}{(C_{17}H_{35}CO_2H)_3}$$

In many bacteria and fungi a lipase is present.

CHAPTER VI.

FERMENTATIONS AND THE ACTION OF ENZYMES—(*Continued*).

1. **Proteins.**—Proteins are among the most important compounds present in the tissues of animals and plants. The majority are exceedingly complex in chemical constitution, and although our knowledge of them has been greatly advanced during the last few years by study of their decomposition products, much remains to be done before the exact nature of these substances is understood. Protoplasm, the peculiar material in the cells of living organisms, with which the phenomena of life are mysteriously associated, consists of an unstable mixture of proteins. They are also found in solutions, or in a colloidal state in blood, lymph, milk, and similar liquids obtained from organic beings. Some proteins are met with in a dense semi-crystalline form in the storage tissues of seeds and tubers, and others have been obtained in a true crystalline state from blood, egg-albumen, and certain seeds; most of them, however, are slimy, non-crystalline substances, like the white of an egg, and contain nitrogen, and sulphur in addition to the elements carbon, hydrogen and oxygen; certain proteins, such as the caseinogen of milk, contain phosphorus also.

The amount of carbon present is usually between 52 to 55 per cent.; nitrogen, 16 to 18 per cent.; hydrogen, 6.5 to 7.5 per cent.; sulphur, from 2.5 to 5.3 per cent.

Many proteins are precipitated as a coagulum or insoluble clot when their solutions are boiled.

They are also peculiar in behaving both as acids or bases. Some of them, such as casein, have the acid character most strongly marked, and form neutral salts with alkalis, while in others the basic properties are most obvious. In all, however, both properties are present in greater or less degree.

It is necessary to note here that much confusion exists in regard to the nomenclature used for the proteins and their derivatives. The term *proteid* is very often used in this country as equivalent to protein, but in Germany it has a more restricted meaning, being applied to a special group of proteins mentioned below; if used at all it should be applied in the latter sense, and brought into line with continental usage. Proteins are not infrequently spoken of as *albumens* by some writers, and in France they are described as "substances albuminoides." The older school of chemists have kept the word *albuminoid* as a term for proteins in general, speaking of albuminoids of milk, feeding cake, etc., while physiologists have habitually used it for a small class of proteins, of which gelatine of bone and keratin of horn are representatives.

2. The classification and nomenclature of the proteins is still in a transition state, and will need revision from time to time to bring it into harmony with the advances made in the knowledge of the chemical and physical constitution of these substances. The following classes and names are those at present recommended by a committee of the Chemical Society of London:—

(1) *Protamines.*—These are strongly basic bodies, obtained chiefly from the spermatozoa of fishes, and are among the simplest proteins. They are not coagulated

by heat, and are unaltered by pepsin, although they break down under the influence of trypsin. Protamines contain neither phosphorus nor sulphur, but have a high nitrogen content (20 to 25 per cent.)

(2) *Histones* are also basic substances, not coagulated by heat, very soluble in acids, but precipitated by ammonia. They are mostly obtained from spermatozoa of fish, the thymus glands of some animals, and from red blood-cells of birds.

(3) *Albumins.*

(4) *Globulins.*—These two groups include the commonest and most representative proteins met with in animal and vegetable tissues. Both are coagulated by heat, and contain sulphur but little phosphorus. Albumins present in egg-white, blood serum and milk are soluble in water, and can be salted out from their solutions by saturating the latter with ammonium sulphate. Globulins are more numerous than the albumins, and are present in eggs, blood, milk, most animal and vegetable tissues, and in many seeds. They are insoluble in distilled water and weak acids, and are readily precipitated from solutions by half saturation with ammonia sulphate; magnesium sulphate, which does not throw down albumins, precipitates globulins. They are feebly acid substances, and dissolve easily in alkaline solutions.

(5) *Sclero-proteins.*—This ill-defined class includes gelatine, the most abundant insoluble protein in bone and cartilage and keratin, the chief constituent of wool, hair and hoofs of animals. To these substances the term "albuminoid" is sometimes restricted by physiologists.

(6) *Phospho-proteins.*—The chief representatives of this class are caseinogen of milk, and vitellin, a constituent of the yolk of eggs. They contain from ½ to 1½ per cent. of

phosphorus, and are acid substances, capable of forming definite salts with alkalis. The pure phospho-proteins are insoluble in water, and are thrown down when acids are added to solutions of their salts.

(7) *Conjugated proteins.*—For these bodies German writers adopt the generic term "proteid." They are compounds which contain a protein united with a radical or molecular group, termed a "prosthetic group." The latter is either (1) a nucleic acid, (2) a carbohydrate, or (3) a coloured product. As the peculiar characters of the conjugated proteins are chiefly dependent upon the kind of prosthetic group which they contain, they may be conveniently divided into three sub-classes, viz.: (*a*) Nucleoproteins, (*b*) Glucoproteins, and (*c*) Chromoproteins. On hydrolysis by acids or enzymes a conjugated protein splits into its prosthetic group and a protamine, histone, or other protein.

(8) *Derivatives of proteins.*—When proteins are acted upon by weak acids or enzymes they undergo hydrolysis, some of the products of decomposition being substances which give the reaction of proteins, but are simpler in construction than the original body from which they have been obtained. The chief of these derived proteins are:—

(*a*) *Meta-proteins*, the so-called acid-albumins and alkali-albuminates, formed by the gentle action of dilute acids and alkalis respectively on proteins, belong to this class.

(*b*) *Proteoses.*—A group which embraces albumoses, globuloses, gelatoses, etc. These substances are simpler transformation products of proteins than the meta-proteins. They can be salted out of acid solutions by ammonium or zinc sulphate, but are not coagulated by heat.

(*c*) *Peptones.*—Further hydrolysis of the proteoses

produce simpler bodies. These are the peptones, very soluble substances, which cannot be salted out of their solutions, but are precipitated by phosphotungstic, phosphomolybdic, or tannic acids.

(*d*) *Polypeptides* are a group of products of protein cleavage beyond the peptone stage. The majority are synthetical substances, which cannot here be dealt with.

3. **Colour Tests for Proteins.**—(1) *The biuret reaction.*—A solution of a protein, when made strongly alkaline with caustic potash, gives a red, pink, or violet colour when a few drops of copper sulphate is added. With many albumins the colour is blue or violet, with proteoses and peptones red or pink.

(2) *Millon's reagent* (a solution of mercurous nitrate in nitric acid) gives a pink or dark-red colour when boiled with a protein which contains the oxyphenyl or tyrosine group. The latter is absent from gelatine and certain proteoses and peptones.

(3) *The Xantho-proteic reaction.*—Strong nitric acid, added to a solution of protein and heated, gives a yellow solution or a yellow precipitate. If ammonia or caustic soda is added afterwards, a deep orange or reddish brown colour is produced.

(4) Proteins containing sulphur, when boiled with a solution of caustic soda containing lead acetate, gives a black or brownish precipitate, or the solution becomes dark-coloured owing to the formation of lead sulphide.

(5) *Reaction of Molisch.*—When a few drops of an alcoholic solution of a-napthol is added to the solution of a gluco-protein, and strong sulphuric acid added to the mixture, a violet colour is produced. The sulphuric acid splits off a carbohydrate from the gluco-protein, and converts it into furfurol. The latter then gives a

violet colour with a-naphthol. With thymol the colour is carmine-red.

(6) *Reaction of Hopkins and Cole.*—Prepare a solution of glyoxylic acid by adding sodium amalgam to a strong solution of oxalic acid; filter after the gas bubbles disappear. To a solution of a protein add a little of the above; shake, and then add strong sulphuric acid. A bluish violet colour is produced, which is due to the presence of tryptophane, one of the dissociation products of most proteins, except gelatine and the protamines. Glacial acetic acid which has been exposed to sunlight for some time often contains a small amount of glyoxylic acid; when such acid is added to a solution of a protein, and strong sulphuric acid then poured in, a violet colour is seen at the junction of the two liquids.

4. **"Salting out" of Proteins.**—Most proteins are colloidal bodies, and can be precipitated from their solutions by adding solutions of various inorganic crystalline salts. Some of them are easily salted out in this way, while others are thrown out of solution with difficulty. By using salts of different nature in varying strengths of solution, mixtures of the proteins may be more or less completely separated into their constituents. The method of salting out has been of great service in obtaining pure unaltered proteins for chemical and physiological study. The substances most frequently used for salting out proteins are:—

Sodium chloride
„ sulphate
„ acetate
„ nitrate

Magnesium sulphate

Potassium acetate

Ammonium sulphate

Zinc sulphate

They are used in variable degrees of concentration, the strength of solution necessary for the precipitation of a particular protein being constant for that body. The most efficient of the substances named are ammonium and zinc sulphate, either of which, in saturated solutions, will salt out all proteins and their derivatives, except the peptones.

Certain acids (so-called alkaloidal reagents) precipitate many proteins when used in excess; those most commonly employed are :—

Phosphotungstic	acid
Phosphomolybdic	„
Tannic	„

They are extensively used for the precipitation of proteoses and peptones, the latter of which cannot be salted out from their solutions.

Ex. 45.—Break an egg across the edge of a cup; open the crack, and allow the white to escape into a beaker, keeping back the yolk. Transfer the white to a flask; mix it with about ten times its volume of water, and shake vigorously so as to dissolve as much as possible of the proteins. Use small quantities of the solution of egg-white obtained to illustrate the colour tests for proteins.

(*a*) *Biuret test.*—To 1 c.c. of the egg-white solution add 5 c.c. of strong caustic potash solution and one or two drops of a 1 per cent. solution of copper sulphate; heat slightly, a violet colour is seen.

(*b*) *Millon's test.*—Weigh out 10 grams of strong nitric acid (sp. g. 1.4), add to it 10 grams of mercury; heat, and allow the metal to dissolve; then dilute the liquid with twice its volume of distilled water; mix, and after allowing it to stand for a time,

pour off the clear liquid and label it Millon's reagent. Add a drop or two of fresh white of egg to 5 c.c of Millon's reagent, and boil; a brick red precipitate is obtained.

(*c*) *Xantho-proteic reaction.*—Add 1 c.c. of the egg-white solution to 5 c.c. of concentrated nitric acid, and boil; a yellow precipitate is obtained. Pour off the excess of nitric acid and add ammonia, the precipitate is coloured an orange or brown tint.

(*d*) *Hopkins and Cole's reaction.*—Add about $\frac{1}{2}$ a c.c. of the egg-white solution to 3 to 5 c.c. of glacial acetic acid; pour in carefully some strong sulphuric acid; if no deep purple colour is obtained use a dilute solution of glyoxylic acid instead of the acetic acid.

Ex. 46.—Pour some undiluted white of egg into a test-tube, and immerse the tube in a beaker of boiling water for a minute or two; notice the coagulation.

Try the same experiment with white of egg diluted and shaken with twenty times its volume of tap water. Is the protein coagulated?

Ex. 47.—Break up a piece of ripe Stilton or Camembert cheese in a mortar with a little water.

Wash out the whole into a beaker with distilled water and filter.

Try the colour tests for proteins in this extract by methods described in Ex. 45.

In what respects do the colour reactions differ from those given with egg-white?

Ex. 48.—Dissolve a gram or two of gelatine in 50 c.c. of boiling water; cool and try the protein colour tests with small portions of this solution.

Compare results with those obtained with egg-white and cheese extract.

Ex. 49.—Try the colour tests with a solution of Witte's peptone and compare results with those obtained with other substances used in previous examples.

Ex. 50.—Shake up 10 grams of flour in a flask with 50 c.c of water and allow it to stand for six hours; filter.

Heat some of the filtrate and note the coagulation of a vegetable albumin. In other portions of the watery extract try the colour tests for proteins.

5. **Hydrolysis of Proteins.** — When proteins are boiled with acids or alkalis, fused with caustic potash or subjected to the action of superheated steam, they are decomposed into simpler substances which do not give the biuret reaction. By the hydrolysing action of boiling hydrochloric or sulphuric acids the protein molecule is split up into a great variety of degradation products, the majority of which are amino-acids. Of these some twenty or more different kinds have been isolated, the chief of which, mentioned below, are found among the dissociated products of practically all except the simplest proteins.

(1) The monamino acids most frequently obtained are: alanine, phenylalanine, leucine, glutamic, and aspartic acids. They are all crystalline bodies with a sweetish taste.

(2) Certain diamino acids are constantly found, namely: arginine, lysine, and histidine. These are strongly basic substances.

(3) Tyrosine, a hydroxyamino acid, is present in nearly all proteins except gelatine and some protamines, and

(4) Tryptophane also. The two last are easily detected in a free or combined state by their colour reactions (see pp. 85-86).

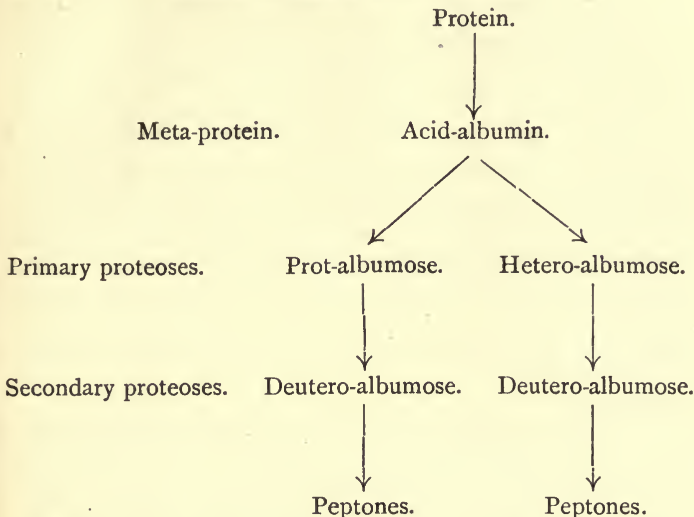
(5) In most cases the sulphur in the molecule is split off as cystine or cysteine.

(6) Ammonia (or its compounds) are also generally met with in the mixture of dissociation products. Possibly in some instances it is a secondary product derived from the decomposition of some of the amino-acids.

The relative amounts of all these substances vary considerably. For example, over 22 per cent. of leucin is obtained from the hydrolysis of egg-white, from casein 10 per cent., while gelatine yields only about 2 per cent. of this substance. The amounts of glutamic acid from casein is about 10 per cent., from gelatine 14 per cent., from serum globulin about 2 per cent.

6. **Proteases or Proteolytic Enzymes.**—Soon after food is taken into the stomach of an animal a colourless, acid liquid, the gastric juice, is poured out from the gastric glands. This juice contains among other substances two enzymes, namely, *pepsin* and *rennin*, and a small amount (.2 to .5 per cent.) of hydrochloric acid. The complex, insoluble proteins present in the food are very soon acted upon by the pepsin, with the result that they are broken down, step by step, into a series of soluble proteins of simpler chemical constitution which can readily diffuse through animal membranes and be transformed into materials subsequently used in building up the body and repairing its waste. Such chemical decomposition is spoken of as *proteolysis*: the various stages of the process are indicated in a general way below.

With Pepsin in acid solutions.



The primary proteoses derived from the acid albumin can be salted out by saturating the solution with sodium chloride or half saturation with ammonium sulphate. Of these two types exist, namely, proto- and hetero-compounds; the former are very soluble in pure salt-free water, while the latter are only very slightly soluble in water and begin to coagulate and separate from the proto-albumoses when the mixture is dialysed to get rid of the salt. The hetero-albumoses are precipitated from salt solutions by dilution with water.

The secondary proteoses, representing products of further hydrolysis, may be precipitated by complete saturation of their solutions with ammonium sulphate.

From the proteoses peptones are ultimately formed. These are bodies of simpler constitution which cannot be

salted out of their solutions, but are precipitated by phosphotungstic acid and other alkaloidal reagents, and give a pure red biuret reaction. They are acid subtances, very soluble in water, and contain no sulphur.

Only very small amounts of ammonia and amino-acids, if any, are produced even by prolonged peptic digestion of proteins, but in many instances dissociation products intermediate between peptones and amino-acids are formed in considerable amounts: these are probably peptides, bodies which on hydrolysis with acids give leucine and other amino-acids.

Ex. 51.—Take three large test-tubes, label them A, B, and C respectively.

Half fill—

A with a dilute solution of hydrochloric acid (1 c.c. of strong acid in 150 of water).

B with dilute solution of hydrochloric acid, to which a few drops of pepsin solution has been added.

C with dilute solution of pepsin only.

Into each tube add a small piece of fibrin which has been washed and allowed to soak for half an hour in distilled water.

Place all three tubes in a water bath kept at 40° C. for twenty to thirty minutes. Examine them at intervals and note that in A and C, in which the ferment and acid act singly upon the fibrin, the latter is undissolved: in B, where the ferment and acid act together, the fibrin is digested and dissolved.

With the solution in B try the following experiments:—

(*a*) To a small portion of the liquid add a few drops of litmus and then neutralize with very dilute caustic soda: *acid-albumin* is precipitated.

(*b*) To another small quantity of the liquid add a drop of strong nitric acid: a white precipitate of a *proteose* or *albumose* appears, which dissolves with yellow coloration on heating but reappears when the liquid is cooled.

(*c*) To a third portion of the liquid add a drop of a 1 per cent. solution of copper sulphate and a few drops of strong caustic soda solution: a pink colour shows the presence of *peptone* (the biuret reaction).

Ex. 52.—Repeat the above Ex., using pieces of the coagulated protein of a hard-boiled egg instead of fibrin.

Ex. 53.—To 10 c.c. of milk in a test-tube add a few drops of pepsin solution and a few drops of dilute hydrochloric acid (see Ex. 51). Keep the whole for two hours in a water bath at 40° C. Note that the milk is at first curdled; after a time the curd is gradually digested and dissolved, the liquid becoming yellowish and bitter.

Filter some of the liquid and test it for peptones (the biuret test).

The enzyme pepsin, which can be easily extracted from gastric juice, acts only in an acid solution.

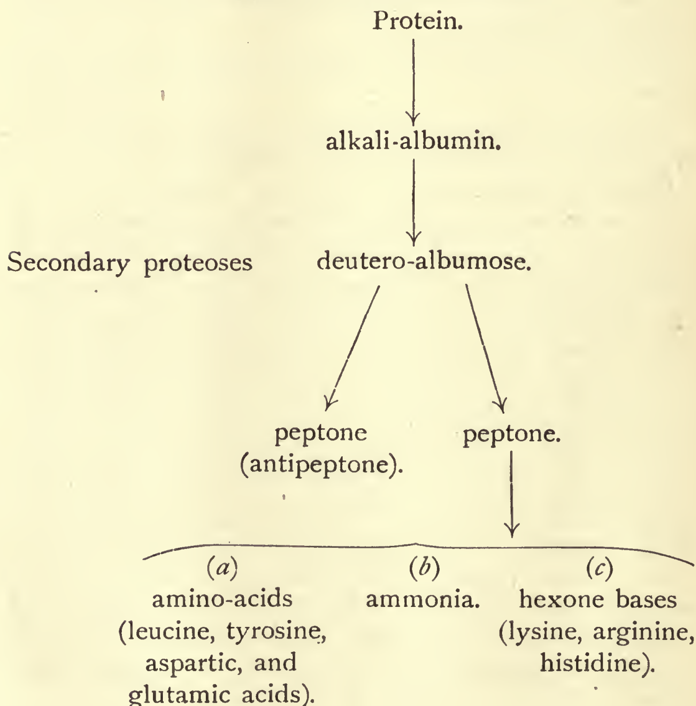
Proteins which escape or are not changed by it, as well as those of simple constitution produced by its action, pass along the intestinal tract and become subjected to the action of the pancreatic juice poured out by the pancreas into the duodenum. This secretion contains several different enzymes, one of them being *trypsin*, a proteolytic ferment, which breaks down proteins best in a faintly alkaline solution. It not only forms soluble proteoses and peptones from proteins but carries on the digestion or splitting of all these compounds further, the peptones being decomposed into several kinds of amino-acids (the chief of which are glycocoll, alanine, phenylalanine, leucine, aspartic, and glutamic acids and tyrosine), a characteristic substance named tryptophane and small quantities of ammonia: none of the latter products are of protein nature, and consequently do not give the biuret reaction.

It is found that trypsin cannot dissociate the whole of the protein into the amino-acids and other simple substances just mentioned; a certain amount of a resistant residue remains after the enzyme has acted for many months.

The action of trypsin is more rapid and complete than pepsin; few or no primary proteoses are obtained, secondary compounds being apparently produced directly.

The action may be represented thus:—

Trypsin (in alkaline solution).



The amount and kind of each of the decomposition products produced by these proteolytic enzymes are dependent upon the nature of the protein attacked and on the temperature, acidity, or alkalinity, and other factors of the reaction.

From the peptic digestion of casein a proteose (caseose) is obtained which contains all the phosphorus of the casein, later peptones arise, as well as paranuclein and paranucleic acids.

With trypsin it is broken down more rapidly into caseose, caseone (an antipeptone residue), tyrosine, and some other simple products.

Gelatine is converted into primary and secondary gelatoses, gelatine peptones, and, according to Nencki, a certain amount of leucine, glycocoll, and ammonia.

Ex. 54.—Take three large test-tubes and label them A, B, C respectively.

Half-fill each with a 1 per cent. solution of sodium carbonate and add five or six drops of Benger's Liquor pancreaticus, which contains trypsin.

Boil A, and then cool it.

Add a drop or two of hydrochloric acid to B, so as to make it acid instead of alkaline.

Leave C alone.

Then add to each a piece of fibrin which has been washed and soaked for half an hour in 1 per cent. sodium carbonate solution.

Place the tubes in a water bath at 40° C. for half an hour.

Examine them at intervals and note that in A no change is observable, the ferment having been destroyed by boiling.

In B digestion does not occur, the solution being acid.

In C the fibrin is eaten away and dissolved without swelling up, as in pepsin digestion.

(*a*) Filter the liquid in C and test a small portion for *alkali-*

albumin by neutralizing it with a few drops of very dilute hydrochloric acid; a precipitate indicates alkali-albumin.

(*b*) Filter off the precipitate and test the filtrate for *peptone* by the biuret reaction.

(*c*) Carefully neutralize the solutions in A and B, then filter and examine the filtrates for peptones.

Ex. 55.—Pour 2 c.c. of milk into a test-tube and dilute with about 10 c.c. of water; then add two or three drops of Benger's Liquor pancreaticus. Immerse the tube in a water bath at 40° C. for half an hour.

Note that the milk is first curdled; the curd is soon digested and dissolved more rapidly and completely than when pepsin is used (compare Ex. 53).

Test the yellowish liquid for peptones by the biuret test.

7. **Proteases or Proteolytic Enzymes of Bacteria.**—In 1887 Bitter found that the cholera bacillus when grown in a liquid nutrient medium produced and set free in the liquid an enzyme which was capable of digesting fibrin and gelatine. Since that date a large number of kinds have been found to secrete or form proteases which break down gelatine and other proteins into simpler compounds. In the ordinary cultivation of bacteria the presence of these proteolytic enzymes is indicated by the liquefaction of the solid gelatine media upon which the organisms are grown. The gelatine is decomposed into peptone, amino-acids, and other simpler substances which dissolve in the water present in the jelly. Where extracellular proteases are produced, that is, where they are manufactured by the living cells and set free into the surrounding medium, liquefaction proceeds at a rapid rate. Common "liquefiers" are, *Bs. subtilis, Bact. prodigiosum*, *Bact. vulgare*, *Bs. vulgatus*, and *Bact. fluorescens.* A number of species are known which only liquefy

gelatine in cultures which have been growing for a considerable time. These form intracellular enzymes which diffuse very slowly, if at all, from normal living cells, but escape readily from unhealthy involution forms or cells which are dead.

The enzymes manufactured by bacteria chiefly resemble trypsin. Like the latter, their action is generally most energetic in neutral or alkaline media, and the majority of them break down proteins into peptones, amino-acids, ammonia, and other basic substances. They all peptonise and liquefy gelatine, some of them even when the medium is faintly acid. Emmerling and Reiser investigated the action of *Bact. fluorescens liquefaciens* on gelatine, and found a peptone present in the "liquefied" medium along with methylamine, trimethylamine, choline, and betaine; ammonia was also set free. *Streptococcus longus* cultivated under anaerobic conditions in a medium containing fibrin produced tyrosine, leucine, trymethylamine, methylamine, ammonia, and some pyridine bases, as well as acetic, propionic, butyric, and succinic acids.

The production of enzymes by bacteria and the amount obtained depend upon the kind of organism, the composition of the nutrient medium, temperature, and other factors. A few species, such as *Bs. subtilis*, *Bact. prodigiosum*, and *Bact. pyocyaneum*, can form them in nutrient solutions devoid of organic nitrogenous compounds, but the majority only give rise to enzymes when grown in meat juice, broth, milk, and other media containing proteins. Most carbohydrates hinder the formation of enzymes more or less completely without checking the growth and development of the bacteria. Some of the sugars, such as glucose, may inhibit their

production altogether; on the other hand, lactose has little effect. A small amount of quinine can prevent enzyme formation by *Bact. pyocyaneum* without interfering much with the growth of the organism.

Although the formation of enzymes may be said to be characteristic of certain species, many bacteria lose this power when cultivated for a long time under somewhat abnormal conditions.

To determine the presence of a enzymes in a culture of any organism grown in broth, milk, or other liquid medium, it is necessary first to remove the living organisms or check their development, after which the action of the solution may be tested upon gelatine, fibrin, or other protein.

By filtration through a Chamberland porcelain filter the bacteria can be removed without destroying the enzymes which they may have secreted; this method is very generally adopted in the study of bacterial enzymes.

Another plan is to destroy the vitality of the organ isms by heat; but as most enzymes are decomposed or their powers greatly diminished at 65 to 70° C.—a temperature which is insufficient to kill many forms of bacteria—the method is unsatisfactory or only of limited application.

A simpler device, and one which is of general use, is to treat the culture with an antiseptic substance of sufficient strength to inhibit the growth of bacteria without affecting the enzyme present. The compounds best serving this purpose are toluene, phenol, thymol, and salicylic acid; chloroform is also useful, but is volatile at somewhat low temperatures.

Ex. 56.—Inoculate two or three tubes of nutrient broth with

Bs. subtilis, *Bact. prodigiosum* or *Bact. liquefaciens* and inoculate for three days at 30° C.

To the cultures add about .25 c.c. of toluene and a very small amount of barium sulphate powder so as to render the liquid turbid: shake thoroughly.

Pour some of this culture into a tube containing ordinary nutrient gelatine which has been made slightly alkaline with sodium carbonate.

Allow the whole to stand and when the insoluble barium sulphate has fallen on the surface of the solid gelatine mark the point on the outside of the tube with a piece of gummed paper.

Keep the tube at 20° C.

If the solution contains a proteolytic enzyme, the solid gelatine will be slowly dissolved and the thin layer of barium sulphate will descend in the tube.

Try the experiment with *Bact. coli* and *Str. lacticus* and note the results.

Ex. 57.—Cultivate one of the bacteria mentioned in the previous Ex. in nutrient broth.

Add .25 c.c. of toluene, shake thoroughly and then suspend in the solution a small piece of Grübler's "Karmin fibrin."

If the culture contains a enzymes the fibrin dissolves and the carmine with which it is stained diffuses and colours the solution.

Try the experiment with *Bact. coli*, *Bact. acidi lactici*, and *Str. lacticus*, and note the results.

Ex. 58.—Melt a tube of nutrient agar in a beaker of boiling water. Pour into it some sterilized skim milk which has been heated to about 50° to 60° C.: the proportion of milk to agar medium should be about one of milk to three of agar. Colo to about 40° C., inoculate with *Bs. subtilis*, *Bact. vulgare*, or other organism which possesses proteolytic powers. Pour into a small Petri dish and inoculate at 30° C.

The casein of the milk is peptonised when the colonies develop, the medium at those points becoming clear.

Try the experiment with *Bact. coli* and *Str. lacticus*.

CHAPTER VII.

FERMENTATIONS AND THE ACTION OF ENZYMES—(*Continued*).

1. **Putrefaction and Decay.**—After the death of animals or plants the materials of which their bodies are composed soon begin to undergo decomposition unless special precautions are taken to prevent it by cooking or the application of antiseptics.

A host of bacteria of many different kinds set up fermentations in the proteins, carbohydrates, fats, and other organic compounds present, and step by step all these substances are broken down into bodies of simpler constitution, some of which have a disgusting odour. Changes go on in all the different classes of compounds, and the whole process is of a very complicated character; it is found, however, that the foul odour is particularly associated with peculiar decompositions taking place in the nitrogenous compounds. To the fermentations of proteins, which are accompanied by the evolution of evil-smelling products, the term *putrefaction* is applied. Changes of this kind not only occur in the organic compounds forming the dead bodies of plants and animals, but also in various products, such as cooked flesh, tinned foods, cheese, milk, and in dung and other materials containing proteins. Putrefaction is most evident when access of air is partially or entirely prevented, *i.e.* when the bacteria causing it are living under

more or less anaerobic conditions; where an abundant supply of air is maintained, as in porous soil or loosely compacted compost and manure heaps, the substances possessing an objectionable odour are either not produced at all, or are rapidly oxidized into odourless compounds, in which case the organic material is said to *decay.* The end-products of the processes of putrefaction and decay are the same, the proteins being finally broken down into simple gaseous bodies such as methane, carbon dioxide, hydrogen, ammonia, and sulphuretted hydrogen, which diffuse into the atmosphere or are washed into the soil by rain.

A great amount of investigation has been carried on to determine the character of the changes involved in the putrefaction process not only in nitrogenous materials exposed to the air, but in food constituents passing through the alimentary canal of animals. Experiments have been made with pure cultures of bacteria upon single proteins, and the general nature of putrefactive degradation is fairly clear. In the first stages the process is similar to that which takes place in proteins under the influence of trypsin or boiling acids, albumoses, peptones, and amino-acids being formed. The splitting, however, proceeds in such a manner that only small amounts of the two former kinds of compounds are met with at any stage of the decomposition. The enzyme trypsin is unable to bring about further change in the amino-acids which it produces, but the putrefactive bacteria can utilize these in their nutritive processes, and carry the degradation much further. From some of the amino-acids ammonia is eliminated, and the corresponding acids without nitrogen are obtained, in the same manner as if they were decomposed by alkalis or

oxidizing agents. Probably in this way are produced the formic, acetic, propionic, butyric, valerianic, caproic, and other similar acids so frequently found in a free state or as ammonium salts in putrefying substances. Cinnamic and oxalic acids are sometimes present.

Two aromatic bodies, p-cresol and phenol, are often obtained also, and are doubtless derived from tyrosine.

The most characteristic substances produced in the putrefaction of proteins by bacteria are indole and skatole, two compounds to which the unpleasant odour of fæces is due. These are derived in some instances directly from the proteins, but they are also produced from tryptophane. The amount obtained is very variable, and is dependent upon the species of bacterium causing the decomposition, the nature of the medium in which it is grown and certain factors which are not understood. Some bacteria, such as the cholera organism and *Bact. vulgare*, manufacture considerable amounts of indole when grown in peptone solutions, while others give rise to little or none. The presence of sugar generally interferes with its formation. *Bact. coli* is able to form indole in broth or peptone solutions so long as the liquid is neutral, but none is obtained when the medium is acid, or when much sugar is present. In view of the fact that one species may produce it, and another nearly allied form be devoid of that power, the presence or absence of indole in a culture medium may be used as a valuable diagnostic character in the identification of bacteria. Methods of testing for it are given in Ex. 63.

From among the products of putrefaction a number of weak bases have been isolated, some of which are crystalline compounds, resembling the vegetable alkaloids : they are known as *ptomaines*. Those of common occur-

rence are various methylamines, ethylamines, certain diamines, such as cadaverine and putrescine, and choline and betaine. Most of them are harmless compounds or only poisonous when taken in large amounts. Many, however, are known which are exceedingly dangerous even in minute doses, and the consumption of putrid or decomposing foods containing them not infrequently leads to fatal results. Muscarine, which has been obtained from putrefying fish, neurine from decomposing flesh, and tyrotoxicon from rotten cheese, are examples of this poisonous class. Similar bodies have been isolated from decaying shell-fish, tinned sardines, and other foods which have undergone putrefactive changes.

It may be noted here that certain pathogenic bacteria, such as the bacilli of tetanus and diphtheria, when grown in culture solutions, are able to produce or secrete much more deadly substances than the ptomaines just mentioned. These are termed *toxins*, but little is known about their chemical nature: some of them appear to be proteoses, while others resemble enzymes.

Sulphuretted hydrogen and methylmercaptan are generally present in putrefying substances, and are derived from organic sulphur compounds such as cysteine, although in some instances bacteria are able to reduce sulphates with the formation of hydrogen sulphide. What becomes of the phosphorus of proteins is not clear. It is said to be given off from decaying fish and other organic materials as phosphoretted hydrogen, but there is some doubt about this statement.

2. **Putrefactive Organisms.**—Although experiments with pure cultures have shown that a great many species of bacteria are able to bring about putrefactive decomposition in materials containing protein, many of them

are rare and not likely to occur in putrefying substances under ordinary conditions. A small number, however, are very widely distributed in nature, and are almost invariably concerned in the destruction of animal and vegetable organic matter. The chief of these are described below.

1. **Bacterium vulgare**, L. and N. (*Proteus vulgaris*, Hauser).—An examination of a piece of putrid meat which has been freely exposed to the air reveals the presence of immense numbers of minute bacteria which were formerly spoken of as *Bacterium termo*. Investigation by Hauser and others showed that these organisms were remarkable in being able to assume very many different growth-forms, occurring sometimes as short rods almost like cocci, and under other conditions as long straight rods or spiral threads. On account of its variability the genus was given the name *Proteus*, and three more or less distinct forms were described under the names *Proteus vulgaris*, *P. mirabilis*, and *P. Zenkeri*. More recently these have been grouped together as varieties of the species *Bacterium vulgare*, L. and N. (*Bacillus vulgaris*, Mig.). They are very common in putrid flesh, and of frequent occurrence in water contaminated with decomposing animal matter. They are also common inhabitants in the intestines of man and other animals.

The following are the chief characters of typical *Bact. vulgare*:—

The organisms are usually slender, very active motile rods, from 1 to 4 μ long and .4 to .7 μ thick. As stated above they may occur as rods 40 μ long or as long spiral threads. They generally stain by Gram's method. Spores are not formed.

Bact. vulgare grows best under aerobic conditions, though it is able to thrive as an anaerobe. From proteins it produces indole and sulphuretted hydrogen, and its cultures in gelatine or bouillon have a foul odour.

Gelatine.—The colonies are at first thin, grey, and semi-

transparent; later the gelatine is rapidly liquefied, the colonies producing shallow cup-like depressions in twenty-four hours or less at 22° C. The stab is thread-like at first; afterwards a cylinder of turbid liquefied medium is seen, with a cup-shaped depression at the surface.

Agar.—The colonies on agar are thin, bluish-grey, and semi-transparent; at first round, but irregular later and extending over the whole surface.

Potato.—Little growth, yellowish-white.

Bouillon.—Turbid throughout, with an abundant sediment in twenty-four hours.

Sugars.—Ferments grape and cane sugars with the production of CO_2 and H_2 (1 : 2); no gas from lactose.

Milk.—Casein is coagulated in two or three days, and afterwards dissolved, with the production of a slightly acid yellow liquid.

Proteus mirabilis, Hauser, is a form of *Bact. vulgare* which readily produces pear-shaped or rounded involution forms 4 to 7 μ in diameter. The colonies on gelatine and agar are similar to those of the typical species, but the former medium is more slowly liquefied.

Proteus Zenkeri, Hauser, is also a variety of *Bact. vulgare*; it does not liquefy gelatine and grows best at 30° C. The surface colonies on gelatine spread out in star-like forms, and the stab shows characteristic thread-like growths all round the track of the needle.

2. Very closely resembling *Proteus Zenkeri* is **Bacterium Zopfii,** Kurth, originally isolated from the dung of fowls, and since found commonly in putrid substances and impure water. It is motile and varies much in form, very short rods and long threads being obtained. Under certain unfavourable conditions the threads break up into round coccus-like cells, which develop again into rods when placed in suitable nutrient media. The colonies do not liquefy gelatine but grow outwards in delicate irregular branches, the whole looking like the mycelium of a

mould. The gelatine stab resembles a miniature inverted spruce fir tree. Bouillon is rendered slightly turbid and a small amount of indole is produced. Milk is not coagulated, and on potato the growth is usually very slight.

3. **Bacterium coli,** Escherich.—Another organism which is usually present in decaying flesh and other organic substances is *Bact. coli commune*, or *Bact. coli* (Esch.) (Fig. 26). It is a constant inhabitant in the intestines of all mammals, and has been isolated from the digestive tract of birds and fishes. The excreta of animals contain it in enormous numbers, and it may be considered as one of the most widely distributed of all bacteria. It is not infrequently met with in milk and other dairy products which have been contaminated with the dung of cattle, and from manured soils and those containing decaying organic debris it finds its way into well and river water. A very large number of allied varieties and forms are known which differ so very slightly from each other that their description and classification is a matter of great difficulty.

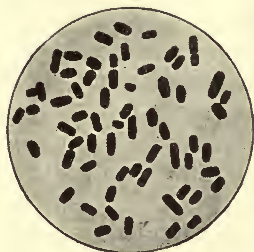


FIG. 26.—*Bacterium coli*, Esch: (×1000).

Typical *Bact. coli* is a short, stumpy bacterium with rounded ends, motile when young, and does not form spores nor stain by Gram's method. It is generally single or united in pairs, each cell being about 1.3 to 3 μ long, and .4 to .5 μ broad It grows best as an aerobe at 37° C., but will thrive under anaerobic conditions, especially when cultivated in media containing sugar.

Gelatine.—The surface colonies on gelatine are moist, translucent, whitish, flat and thin, with an irregular wavy outline, the deep ones being round and yellowish-brown: there is no liquefaction of the medium. In gelatine stab it forms a thick white opaque growth along the track of the needle.

Agar.—On agar the colonies are smooth, shining, greyish-white, elevated and not so irregular in outline as on gelatine.

Potato.—A yellowish-brown growth with puckered elevated outline.

Bouillon.—The whole liquid is soon turbid and a dense sediment is deposited. In peptone water indole is produced and much sulphuretted hydrogen gas.

Milk is rendered acid at 37° C. and coagulated in less than seven days, the curd containing more or less gas.

Sugar.—Grape sugar and lactose are fermented with the production of acids and much gas, about one-third of which is carbon dioxide, the rest hydrogen and nitrogen. It ferments dulcite also, but most forms do not attack saccharose. A variety which ferments and produces gas from the latter sugar is known as *Bact. coli communior.*

4. Associated with *Bact. coli* in the alimentary canal of many animals is **Bacterium lactis aerogenes** or **Bacterium aerogenes,** Escherich, which was first isolated from the small intestine and fæces of infants fed on cows' milk. It is found throughout the intestinal tract and in the stomach, while *Bact. coli* is chiefly confined to the colon and rectum.

Typical *Bact. aerogenes* is a short, thick bacterium very similar in form and dimensions to *Bact. coli*, usually 1 to 2 μ long and .5 μ broad, though much longer cells are not uncommon: it is Gram negative.

Like *Bact. coli* it does not form spores, and is a non-liquefier; it is, however, never motile at any stage of its development.

Gelatine.—On gelatine the surface colonies are rounder in outline, opaque, porcelain-white and convex. The deep colonies are greyish-brown and granular.

Agar.—Thick white shining colonies.

Potato.—Whitish or yellowish brown growth, with elevated warty edges and cheesy odour.

Bouillon.—Turbid, with much sediment, and usually a pellicle or scum on the surface. It forms indole.

Milk.—Milk soon becomes strongly acid, and is coagulated,

the curd having small gas bubbles in it. The organisms when grown in this medium, generally develop capsules.

Sugars.—Glucose, lactose, and saccharose are all fermented with the production of acid and much gas, but it does not ferment dulcite. When *Bact. lactis aerogenes* is grown for three days in 2 per cent. glucose bouillon in a fermentation tube, and a strong solution of caustic potash then added, a pink or reddish fluorescent colorisation is developed after twenty-four hours: this is known as the Voges-Proskauer reaction. (Ex. 65.)

5. Another "coliform" organism frequently met with is **Bact. cloacæ**, Jordan. It is one of the common bacteria in sewage, has been isolated from the intestinal canal, and sometimes occurs in impure water and dirty milk. This bacterium is motile, and in general morphological characters closely resembles *B. coli.* The surface colonies in gelatine are thin, whitish, and translucent, with darker centre and wavy outline; the deeper ones round and yellowish. Gelatine is slowly liquefied in four to thirty days. Bouillon is rendered turbid in twenty-four hours, generally with slight scum formation. Milk is acidified and coagulated in ten to eighteen days. It ferments glucose and saccharose rapidly with acid and gas production, and lactose, also, when the cultivation is prolonged. Dulcite is not fermented by it, and it does not produce indole in peptone solutions; like *B. aerogenes*, however, it gives the Voges-Proskauer reaction.

Other organisms closely allied to *B. coli* are *Bact. Grünthal*, *Bact. neapolitanus*, *Bact. oxytocus perniciosus*, and *Bact. coscoroba.*

For the biological characters of *Bact. coli* and "coliform" glucose-fermenting organisms, see table on p. 306.

6. **Bacterium fluorescens liquefaciens**, Flügge, is one of the commonest species of bacterium in water, soil, and sewage; it also occurs in milk and other food materials sometimes, and assists in the putrefaction of organic substances. It is a motile organism from 1.4 to 5 μ long, .4 to .5 μ broad, with a bundle of three to six polar flagella, on account of which it is occasionally

included in the genus *Pseudomonas*. *B. fluorescens* is markedly aerobic, stains very slightly or not at all by Gram's method, and is somewhat difficult to stain with any dye except hot carbol-fuchsin. It grows rapidly at 20° C., and does not form spores.

Gelatine.—On gelatine the surface colonies are round at first, later somewhat irregular in outline, and producing rapid liquefaction; the deep colonies are pale yellow or greenish yellow. The liquefied material exhibits a characteristic yellowish or bluish-green fluorescence; in it the bacterial colonies are found as flocculi or crumbly masses, which settle to the bottom of the liquid. The gelatine stab liquefies in the form of a cylinder, with funnel-shaped depression at the surface.

Agar.—Colonies moist, rounded, yellowish or greenish, with fluorescent border.

Potato.—Growth moist, slightly raised, yellowish at first, later pale sepia brown.

Bouillon.—Becomes very turbid, with a pellicle on the surface, and shows a striking yellowish green fluorescence; usually only traces of indole are found.

Milk is gradually changed to a yellowish-green alkaline liquid, generally without previous coagulation; any curd which is precipitated soon dissolves.

Sugar.—Glucose bouillon is rendered acid without development of gas.

A number of allied forms are known, which are possibly varieties of one species. The "green" and "blue" pus organism, **Bact. pyocyaneum**, Flügge, a pathogenic species or form commonly associated with pus of wounds which heal unsatisfactorily, is very nearly related, or merely a modification of *Bact. fluorescens liquefaciens.* The two organisms are morphologically alike, and in many physiological characters very closely resemble each other also.

Another organism, **Bact. fluorescens non-liquefaciens**, Eisenberg (*Bact. immobile*, Kruse), resembles the type, but liquefies gelatine very slightly. It is nearly related, if not identical, with *Bact.*

fluorescens putidus, Flügge, frequently isolated from putrefying substances, to which it gives a greenish tint and an odour of trimethylamine. The latter is a motile organism, 1.6 to 5 μ long, .4 to .6 μ broad, with one or two flagella only. The surface colonies on gelatine are transparent of *coli* form, but yellowish-green in colour, and do not liquefy the medium; in other respects the organism agrees with *Bact. fluorescens liquefaciens.*

7. A common inhabitant of putrefying material is **Bs. prodigiosus**, a description of which is given on page 282. It sometimes develops bright red blood-like spots on bread, potato, and other starchy foods.

8. In addition to the organisms previously mentioned certain species of cocci, namely: (1) **Micrococcus pyogenes** (L. and N.), var. **aureus** (*Staphylococcus pyogenes aureus*, Rosenbach); (2) **Streptococcus pyogenes**, Rosenbach; (3) **Micrococcus flavus**, Flügge, are very frequently present in the early stages of the putrefaction of organic substances. All are widely-distributed organisms. The two former are of special importance to the pathologist, since they are responsible for several inflammatory and suppurative diseases in man.

(1) **M. pyogenes** is a coccus from .9 to 1 μ in diameter, and occurs in irregular clumps or in pairs. It is non-motile, and stains by Gram's method. Growth goes on well at temperatures between 27 to 37° C., especially as an aerobe, although it will thrive under anaerobic conditions. The colonies in gelatine are small, whitish or pale yellow, and liquefy the medium. On agar and potato they are whitish at first, but ultimately become a bright orange yellow colour. Milk is coagulated and made acid in a week or less. Bouillon soon becomes cloudy, but finally clears, and deposits a yellow sediment.

Several varieties are met with, some of them differing in the production of lemon-coloured (var. *citreus*) or white colonies (var. *albus*).

(2) **Streptococcus pyogenes** occurs in the form of long chains,

each coccus being about 1 μ in diameter, and readily stains by Gram's method. It is a facultative anaerobe, and grows very slowly at 20° C., the optimum temperature being 37° C. The colonies on gelatine and agar are always very small, round, and white or yellowish white, almost flat, and do not liquefy the medium. On potato there is little or no growth. Milk is generally coagulated in twenty-four hours or less, and glucose bouillon becomes acid without separation of gas. A great many allied forms or species are known, differing chiefly in length of "chains" and in pathological virulence.

(3) **Micrococcus flavus** is a coccus .5 to 1 μ in diameter, occurring usually in pairs, or three or four together. It is an aerobic species, which grows well at 20° C. The gelatine colonies are small, round, yellowish-white; they liquefy and sink into the medium. The liquefied gelatine stab is funnel-shaped, with yellowish sediment. On potato its growth is distinct, thin, and yellowish or greenish yellow. Milk is only partially coagulated, and rendered acid in two or three weeks. This species is said to be identical with or closely allied to *Sarcina lutea*, Flügge, but typical sarcina "packets" are not formed.

9. The spore-producing species, **Bs. subtilis** (page 124) and **Bs. vulgatus** (page 126), are not uncommon bacteria in decaying organic matter.

10. The organisms already described are those chiefly found in the early stages of the putrefactive process, when there is abundant access of air. The production, however, of some of the most characteristic foul-smelling substances in decomposing materials is dependent upon anaerobic conditions, and bacteria, which carry on their work best in the absence of oxygen, are always present in putrefying substances. In fact, the proteins of organic matter appear to be more rapidly and more effectually broken down by anaerobic species than by the aerobes.

The chief of these anaerobes is **Bacillus putrificus**, Bienstock, which is found in dung and sewage, and is very common in

the soil. It is a long, motile, rod-shaped organism, 5 or 6 μ long and .8 μ broad. Very resistant spores are formed at 35° C. which arise at one end of the bacillus, the whole becoming the shape of a drum-stick. *Bs. putrificus* produces a tryptic enzyme, which splits up proteins with the formation of amines, a solution of gelatine being changed into a foul-smelling liquid, with the evolution of much gas, when access of air is prevented.

Ex. 59.—Show the production of ammonia from proteins. Prepare the following solution :—

Di-potassium phosphate . .	5 gr.
Magnesium sulphate . .	2.5 gr.
Sodium chloride . . .	2.5 gr.
Water	1 litre.

Take three 200 c.c. flasks and pour into each 100 c.c. of the solution.

Add to one .1 per cent. of gelatine,
„ another .1 per cent of fibrin,
„ a third .1 per cent. of peptone,
and sterilize.

Inoculate each with *Bs. mycoides* and incubate at 30° C. for ten to fifteen days.

(*a*) Test for ammonia with Nessler's solution.
(*b*) Heat with magnesia, and test the gas given off with litmus paper.

Control tubes uninoculated should be tested also.

Ex. 60.—Add to a litre of the inorganic solution in previous exercise—

Glucose	2 gr.
Sodium nitrate . . .	2 gr.

Sterilize: inoculate 100 c.c. of it with *Bs. mycoides.* Incubate at 30° C., and every second day :—

(1) Test for nitrites (Ex. 74).

(2) Test for ammonia with Nessler's solution.
(3) Find out when the nitrite disappears.

Ex. 61.—To a very dilute solution of egg albumin add a dilute solution of ferrous sulphate to prevent coagulation; sterilize at 100° C. Inoculate with pure cultures of—

(*a*) *B. mycoides*,
(*b*) *B. fluorescens liquefaciens*,
(*c*) *B.* (*Proteus*) *vulgare*,[1]

and incubate at 30° C. for ten to fifteen days.

(1) Test for ammonia with Nessler's solution.
(2) Heat with magnesia and test the gases evolved with litmus paper. Compare with uninoculated sterilized control tubes.

Ex. 62.—Chop in pieces a small piece of beef and place it in a beaker of water.

Leave in a warm place for twenty-four to forty-eight hours until putrid.

Examine a drop of the putrefying liquid for *Proteus* bacteria. Note their lively motion.

Stain some with gentian-violet. Inoculate gelatine and agar plates with a drop of the putrid liquid diluted with sterile water and observe the form of the colonies obtained.

Ex. 63.—The Indole Test.—Prepare a 2 per cent. solution of peptone; add to it ½ per cent. of common salt and make it slightly alkaline with a drop or two of sodium carbonate solution.

Place about 10 c.c. in a number of tubes and sterilize.

Inoculate tubes with—

1. *Bact. vulgare*,
2. *Bact. coli*,
3. *Bact. lactis aerogenes*,
4. *Bact. fluorescens*,

and incubate for six to seven days.

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[1] Pure cultures of these and other organisms can be obtained from the Author.

Test for indole in each—

(*a*) *With sodium nitrite.*—Make a small stock 5 per cent. solution of sodium nitrite. Take 1 c.c. of this and dilute it to 100 c.c. Then add 1 or 2 c.c. of the dilute .05 per cent. solution to the tube to be tested, and pour a few drops of *pure* concentrated sulphuric or hydrochloric acid down the side of the tube.

The acid liberates nitrous acid, which reacts with any indole present, producing a pink or purple colour. If pink colour is absent or not distinct, place the tube for thirty to forty minutes in an incubator at 37° C.

Control tests should be made in uninoculated tubes to show that the reaction is due to the growth of the bacteria.

(*b*) *With paramidobenzaldehyde.*

i. Marshall's test.

Dissolve 6 gr. of para-dimethylamidobenzaldehyde in 100 c.c. of water and add 12 c.c. of concentrated sulphuric acid.

This reagent gives a rose or cherry-red colour when added to cultures containing indole, and is more reliable than the former test.

ii. Ehrlich's test.

Solution A. Dissolve 1 gr. of para-dimethylamidobenzaldehyde in 100 c.c. of 96 per cent. alcohol, and add 20 c.c. of concentrated hydrochloric acid.

Solution B. Saturated solution of potassium persulphate in water. To 10 c.c. of the culture to be tested add 5 c.c. of each of the solutions A and B; shake and allow to stand for five minutes: a red colour indicates the presence of indole.

Ex. 64.—Grow the organisms mentioned in the previous exercise in slightly alkaline sugar-free bouillon.

Incubate for eight to ten days. Then test for indole, as above.

Ex. 65.—The Voges-Proskauer Reaction.—Prepare a 2 per cent. glucose bouillon: place it in fermentation tubes and sterilize.

Inoculate tubes with—

1. *Bact. coli*,
2. *Bact. lactis aerogenes*,
3. *Bact. cloacæ*,

and incubate at 22° C. for three days.

Then add 2 or 3 c.c. of strong caustic potash solution.

The development of a pink colour on standing exposed to the air for a time indicates the Voges-Proskauer reaction. Which of the above give it?

3. **Oxidizing Enzymes or Oxidases.**—When certain fungi are cut open and the cut surfaces exposed to the air the colourless juice or cell-sap of the plant becomes blue, red, brown or otherwise colourized. A brown colour also develops on the cut parts or in the expressed juices of apples, pears, and many other fruits and vegetable tissues. These colour changes are due to the oxidation of certain compounds through the agency of enzymes, which have been termed *oxidases.* They are very widely distributed in the cells of plants and in milk and other animal secretions: they are also produced by many kinds of bacteria as well as by yeasts and other fungi.

Like other enzymes, they act in a specific and restricted manner upon certain definite substances only. All of them behave as carriers of oxygen, yielding the latter element to the substances which are oxidized, and reoxidizing themselves from the air or from other easily reduced compounds. In many cases the "browning" of plant tissues has been shown to be due to reddish or brown compounds, resulting from the oxidation of tannin or tyrosine by oxidases.

Some of them, which may be spoken of as *direct oxidases*, can utilize the free oxygen of the air; others are only able to effect oxidation in the presence of a peroxide, such as peroxide of hydrogen, which readily yields active nascent oxygen; such are termed *peroxidases.*

The presence of oxidases is readily tested by means of an alcoholic solution of gum guaiacum, which when oxidized gives a rich blue colour.

The addition of a direct oxidase results in a blue

colour immediately, but the colour does not develop until hydrogen peroxide is added if a peroxidase only is present.

Ex. 66.—Make a tincture of gum guaiacum by adding a few lumps of this substance to a test-tube or flask containing alcohol; boil for some time, and to the yellow solution add water cautiously until near the point at which a permanently milky emulsion is formed. If too much water is accidentally added, and the solution remains milky, it may be cleared by the addition of a few drops of alcohol.

(*a*) Cut thin slices of apple, potato, and mangel, and place them in small portions of the guaiacum solution.

Note the production of a blue colour.

(*b*) Try the experiment with slices which have been boiled for five minutes.

(*c*) Add the juice from raw beef to some of the solution, and note if a blue colour is produced; if not, try the addition of a few drops of hydrogen peroxide.

An enzyme termed *catalase* is very widely distributed in the protoplasm of all living animals and plants, and is also found in many bacteria. It has the power of liberating oxygen from hydrogen peroxide, but the oxygen set free does not give a blue colour with tincture of guaiacum.

Ex. 67.

(*a*) Pour some hydrogen peroxide solution over the surface of an agar plate culture of several different kinds of bacteria, and note the evolution of bubbles of oxygen. Where are the bubbles most abundant?

(*b*) Try the same experiment, using the tincture of guaiacum and hydrogen peroxide mixture of Ex. 66. Is any blue colour produced?

CHAPTER VIII.

BACTERIOLOGY OF THE SOIL.

1. THE bacteriological examination of the soil is one of the most recent branches of scientific study. A good beginning has been made, but a very extensive unexplored field yet lies before the soil bacteriologist.

The work carried on by certain groups of organisms has been examined in considerable detail, and the processes of putrefaction and decay of organic substances so far as relates to the nitrogen in them are fairly understood. Much is known also of the nature and causes of nitrification, denitrification, and the fixation or assimilation of the free atmospheric nitrogen by soil organisms; but many of the problems connected with the formation and decomposition of humus, the chemical changes induced by bacteria and fungi in the mineral constituents of the soil, and the influence of biological processes upon the physical character of the latter await solution.

Not only is further research needed respecting individual processes going on within the soil, but systematic study of the various kinds of organisms present in it—moulds, algæ, and protozoa, as well as bacteria—is greatly wanted. The relationship of the work done by each group must be exhaustively investigated, and the relative importance of the various biological processes in the production and maintenance of the fertility of

cultivated land are problems which have yet to be dealt with.

2. **Numbers of Bacteria in the Soil.**—It is a difficult matter to determine the number of bacteria in a sample of soil, and no really reliable method exists at present for the accurate numerical estimation of all the various kinds which may be present in it.

The counting of the colonies on agar or gelatine plates, inoculated either with a definite amount of soil or with a measured volume of sterilized water mixed with the soil to be examined, is generally adopted. However, many kinds of soil organisms, and especially those concerned with the fixation of nitrogen and the process of nitrification, do not grow at all on these media, and unless special precautions are taken to exclude air no colonies of the anaerobic forms appear upon the plates.

The method adopted to free the bacteria from the soil particles, the nature and chemical reaction of the media employed, and many other factors influence the result.

The figures given below are comparative only, and have mostly been determined by plate cultures on neutral or slightly alkaline gelatine or agar media.

Very little is known in regard to the relationship between the number of organisms in a soil as determined by the plate methods in ordinary agar and gelatine media and its fertility. That an increase or decrease in the number of bacteria present has an important bearing on the material available for the nutrition of crops is certain, but much research must be prosecuted before the connection is clearly understood.

The soil, containing as it does considerable amounts of water and stable organic matter, is an excellent medium for the development and multiplication of many

kinds of bacteria. The number present in it varies very much, being dependent on a great many factors, the chief of which are the kind of soil, the depth at which the sample is taken, the amount of moisture in it, its physical condition, the time of year at which the examination is made, and the kind of crop grown upon the land and the manuring it has received.

Remy found in one gram of arable soil in fair condition from 6 to 10 millions of bacteria and half a million moulds, while in a poor sandy soil there were only 200 to 450 thousand bacteria and from 45 to 168 thousand moulds.

Other investigations have shown that from 1 to 5 or 6 millions may be expected in most ordinary soils.

The bacteria are chiefly met with near the surface of the ground, *i.e.* within the first 9 to 12 inches, where good aeration and a suitable degree of moisture and temperature prevail. Below this the conditions favourable for bacterial life are absent, and the numbers diminish rapidly with increasing depth until a point is reached where the soil is sterile.

Houston found about 1,688,000 in a gram of surface soil, at a depth of 1 foot 1,100,000, at 3 feet deep 174,000, and at 6 feet below the surface only 410. Other examinations have shown that in some soils few or no living micro-organisms were met with below about 3 or 4 feet. Where the soil is of open texture and contains large amounts of organic compounds the number increases, and more bacteria are able to penetrate and live in the deeper layers than where the soil consists of poor compact clay.

The number of bacteria appears to go up and down with variations in temperature at different seasons of the

year, but careful observations have shown that this is more due to alterations in the degree of moisture of the soil than to temperature changes. The experiments of Engberding and others indicate that the optimum development of the majority of organisms occurs when the soils contain 70 to 80 per cent. of their maximum water-holding capacity.

Dry soils contain few bacteria, and dryness has been shown by Löhnis to have an injurious effect on nitrification and nitrogen assimilation.

Acid peaty soils and marsh land are poor in bacteria. After drainage the number increases only slightly, but by cultivation and the application of lime, sand, clay, and dung the bacterial content soon approximates to that of ordinary cultivated soils.

The following figures of the change in the number of bacteria after draining and cultivation of "ling, cotton grass, and sphagnum moor" in Sweden are given by Fabricius and von Feilitzen :—

	Virgin moor.	Drained.	With addition of sand and lime.
In May, .	56,000	204,000	4,797,000
July, .	225,000	384,000	8,129,000
Sept, .	144,000	64,000	7,771,000

Farmyard dung and other organic manures contain enormous numbers of bacteria and large amounts of compounds upon which bacteria thrive. After these are applied to the soil the numbers, especially of putrefactive and denitrifying organisms, rapidly rises for a time. Heavily manured soils, or those to which sewage has access, may contain from 50 to 120 millions per gram. Later, however, the number falls to the original

figure, the normal soil bacteria finally getting the upper hand in the struggle which ensues.

No doubt the food material suited to *B. coli* and other allied putrefactive forms soon diminishes, and there is probably also an accumulation of organic acids and other compounds inimical to the growth of this class of bacteria.

The ploughing in of weeds, stubble, and "green manures," which contain organic nitrogenous and carbon compounds, provides pabulum for soil organisms and assists their development.

Certain forms of *Streptothrix* and many moulds are increased by the addition of straw, and probably the organisms which "fix" free nitrogen are increased also by straw residue.

Ammonium sulphate and nitrate of soda can be utilized by many soil bacteria for nutritive purposes, and potash phosphates and lime have been found to assist in the development of the nitrogen assimilating group of bacteria.

The growth of certain crops favour the development of bacteria in the soil more than others. Stohlau and Ernest in one case found where clover had been grown seven or eight millions present in a gram of the soil, but only one to two millions per gram of soil from an adjoining plot after barley.

Ex. 68.—Prepare the following "Heyden-Agar" medium :—

Agar, . .	12.5 gr.
Albumose, (Heyden) .	7.5 gr.
Distilled water, .	1 litre.

Dig up a block of soil with a spade ; break the block, and from the centre take out a small sample and place it in a sterilized bottle.

From this weigh out 15 or 20 gr. and add it to 300 or 400 c.c. of sterilized tap water in a ½-litre flask. Shake well, and if necessary break up the soil with a sterilized glass rod.

Take up with a wide bored sterilized pipette 25 c.c. of the turbid water, and add it to another flask containing 300 or 400 c.c. of sterilized tap water.

From this second flask take 25 c.c. and transfer to a third, and from the third to a fourth flask.

A. Inoculate a Petri dish of the Heyden agar medium with 1 c.c. of the water from the fourth flask and incubate at 20° C. Count the colonies which appear in ten days, and calculate the number of bacteria present in 1 gr. of the original soil.

B. Inoculate a Petri dish of—

(i) Nutrient gelatine.

(ii) Nutrient agar neutralized or rendered very slightly alkaline with a drop or two of sodium carbonate solution, with 1 c.c. of the "soil-dilute," as in *A.*

Incubate at 20° for ten days.

Examine every day and count the colonies. Use a Pake's disk (Fig. 45) beneath plate to facilitate counting.

The colonies of liquefiers on the gelatine medium may be killed by touching them with a "caustic pencil" or stick of silver nitrate.

Compare the results obtained with the three media.

Ex. 69.—Repeat the above with—

(i) Soil from a well-manured garden.
(ii) „ an arable field.
(iii) „ an old pasture.

3. **Kinds of Bacteria in the Soil.**—A large number of kinds of bacteria have been isolated from the soil. Some of them are added to it in the manures which are applied to the land, others find their way into it in drainage water, sewage, and rain. Many of the species are casual introduc-

tions, whose presence is dependent more or less upon accident. Others there are which must be considered as normal inhabitants of the soil. To the latter class belong many of the putrefying and denitrifying organisms, as well as the nitrifying and nitrogen-fixing bacteria which are described in the succeeding pages. The number of these found by Löhnis in samples of soil taken from a field in January and July respectively were :—

	Jan.	July.
Putrefactive bacteria, . .	3,750,000	5,000,000
Urea-splitting „ . .	50,000	50,000
Nitrifying „ . .	7,500	2,500
Denitrifying „ . .	50,000	50,000
Nitrogen-fixing „ . .	1,125	750

Certain anaerobic spore-bearing forms are frequently met with also. Among the bacteria of especially common occurrence in most field and garden soils may be mentioned :—*Bs. mycoides*, *Bs. subtilis*, *Bs. vulgatus*, *Bs. megatherium*, *Bact. fluorescens liquefaciens*, *Bact. fl. non-liquefaciens*, *Bs. putrificus*, *Micrococcus candicans*, various forms of *Proteus*, and certain species of *Cladothrix*.

Bact. coli is commonly present in soils which are polluted with sewage, or to which dung and similar organic excreta have been recently added, but is not usually found, or only in relatively small numbers, in virgin soils.

In addition to these, certain pathogenic species, such as *Streptococcus pyogenes*, *Bs. tetani*, and *Bs. œdematis maligni*, are not uncommonly met with in the soil.

Gottheil made an extensive study of the spore-bearing bacteria attached to the roots of carrot, beet, radish, turnip, parsley, cabbage, and other plants, and found

Bacillus asterosporus, *Bs. ellenbachensis*, *Bs. graveolens*, *Bs. pumilus*, *Bs. ruminatus*, *Bs. tumescens*, and a form of *Bs. subtilis* were very frequently associated with roots of the plants mentioned.

Whether a form of symbiosis exists between the higher plants and these organisms is not at present clear.

Below are descriptions of the chief common organisms present in almost all soils.

Bacillus subtilis, Cohn (Fig. 27), commonly known as the "hay bacillus," is found in hay and in almost all soils. It is a stout rod-shaped organism, from 3 to 9 μ long, .8 to 1 μ broad, rounded at the ends, motile, and often united into long chains. *Bs. subtilis* is strongly aerobic, and produces oval spores 1.7 to 2 μ long and .8 broad, which germinate from the side (equatorial germination).

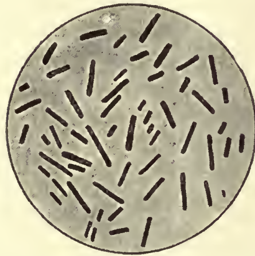


Fig. 27.—*Bacillus subtilis*, Cohn (×850).

The organism stains by Gram's method.

Gelatine.—The surface colonies soon liquefy, producing a cup-shaped depression, filled with greyish liquid. Examined with a low power (× 60), very young colonies are yellowish and round, the edges usually fringed with hair-like extensions.

In gelatine stab the liquefied portion is cylindrical, greyish-white, with a firm white skin on the surface.

Agar.—The surface colonies are small, greyish-white, and, with a low power (× 60), seen to have very ragged irregular edges, these parts resembling tangled threads of cotton. The deep colonies generally have a more tangled appearance than those on the surface.

The stab is thread-like, with one elevated round, moist growth at the surface of the medium, greyish in colour.

Potato.—Thick, white or yellowish growth, dull, becoming mealy in older cultures.

Bouillon.—The liquid soon becomes turbid, and a skin forms on the surface or becomes attached to the sides of the tube.

Milk.—Milk is rendered strongly alkaline, and is coagulated, the curd eventually dissolving: a pellicle forms on the surface.

Sugar.—Solutions of dextrose and saccharose are made acid.

Nitrate bouillon.—The nitrates are rapidly reduced to nitrites and a pellicle forms.

Bs. mycoides, Flügge, sometimes spoken of as the root bacillus, is a very common inhabitant of most soils.

It is a stout rod-shaped bacillus, 6 to 12 μ long and about 1.2 μ broad, with almost square ends, only slightly motile as a rule. It forms chains, is aerobic, and stains by Gram's method.

The spores are oval, 1.5 to 2 μ long, .8 to .9 broad.

Gelatine.—The colonies appear to be formed of fine extraordinarily tangled threads, sometimes in the form of a circular patch, but soon ramifying outwards in all directions, and very much like a growth of mould. The medium is, however, soon liquefied.

The stab is whitish and characteristic, the growth extending from the liquefied track in the form of delicate hairs arranged parallel to each other. On the surface a felted membrane is formed.

Agar.—The surface colonies in agar are at first round and greyish, tangled at the edges. The stab has hair-like extensions, like those in gelatine.

Potato.—The growth on potato is abundant and very closely resembles that of *Bs. subtilis.*

Bouillon soon becomes turbid, with a thick pellicle on the surface; later a sediment is formed and the liquid clears.

Milk is made alkaline and the curd peptonized.

Bs. megatherium, De Bary.—This organism was originally isolated from infusions of cabbage leaves and is commonly

present in soil. It is a large bacillus, 5 to 20 μ long, 1.5 to 2 μ broad, sometimes slightly bent and united in chains; the ends are not rounded.

It moves with a somewhat sluggish motion, is aerobic, and produces large spores, 2 to 2.7 μ long and 1 μ broad, which exhibit polar and equatorial germination.

Stained by Gram's method.

Gelatine.—The colonies are round at first, later kidney-shaped, generally without the fringed edge so frequently seen in *Bs. subtilis.* The medium is liquefied.

The stab shows tube-like liquefaction, the liquid being turbid.

Agar.—Greyish elevated colonies, somewhat granular, with short ramifications round the edges, capable of being drawn into threads, and more slimy than those of *Bs. subtilis.*

Potato.—Like *Bs. subtilis,* but usually yellower and more mealy.

Bouillon is made turbid.

Milk.—Curd precipitated with very slight acid reaction at first; later slightly alkaline with little peptonization of the casein; no pellicle is formed.

Nitrate bouillon.—The nitrates are not reduced, and no pellicle is formed.

Bs. ellenbachensis, *a,* Caron.—The "Alinit-bacillus" (see p. 183). The bacillus closely resembles *B. megatherium,* and according to some authorities is a form or variety of this organism. It is rod-shaped, 3 to 6 μ long, 1.25 to 1.5 μ broad, often single or united in pairs. Few or no long chains are formed. It is motile, produces spores which germinate at the poles, and is stained by Gram's method. Its behaviour on the various media is very similar to that of *Bs. subtilis.*

Bacillus vulgatus, or **Bs. mesentericus vulgatus,** Flügge.—This organism is ordinarily termed the "potato bacillus," growths of it being frequently found on imperfectly sterilized potato slices. It is a common soil bacteruim, usually shorter and more slender than *B. subtilis,* being 2 to 7 μ long and .8 μ broad,

frequently united into short threads. It is aerobic, motile, stains by Gram's method, and forms small spores 1 to 1.2 μ long and .6 broad.

Gelatine.—Surface colonies round, with greyish-white crumpled skin, which soon sinks in the liquefied medium. The edges of the colonies are not finely radiated as in *Bs. subtilis*, but are seen to be irregularly lobed when magnified ($\times$ 50).

The stab liquefies in the form of a narrow tube, the surface being covered with a greyish membrane.

Agar.—Surface colonies greyish, round, moist, and slightly elevated, becoming drier and wrinkled; deep colonies, round or spindle-shaped.

Potato.—The growth is abundant, usually greyish or yellowish, glistening, and very much crumpled and twisted. Some varieties produce a pink, brown or blackish colour.

Bouillon.—Slightly turbid, with a strong whitish film on the surface.

Milk is generally coagulated slowly, and rendered alkaline and slimy; the curd is peptonized after a time.

Micrococcus candicans, Flügge, is a coccus very widely distributed in the air, water, and soil. The cocci are 1 to 1.2 μ in diameter, usually single or collected in small irregular clumps. It is an aerobic organism, grows well at room temperature, and is stained by Gram's method.

Gelatine.—The surface colonies are non-liquefying, round, in about a week 2 to 3 mm. in diameter, raised, moist, and milk-white.

The deep colonies are small, .3 to .5 mm. in diameter, round or lens-shaped, and opaque.

The stab is thread-like and beaded.

Agar.—The colonies are similar to those in gelatine, but whiter and more raised.

Potato.—A dense white, oily growth with wavy margin, extending little.

Bouillon.—Generally very turbid at first, ultimately becoming clear with sediment and a surface pellicle.

Milk.—Becomes slightly acid after two or three weeks without coagulation.

Cladothrix dichotoma, Cohn.—This organism belongs to Chlamydobacteriaceæ, a family of organisms included among the bacteria, but showing much affinity with the blue-green Algæ. It is met with in the form of long cylindrical threads from 1 to 3 μ in diameter and 1 to 2 mm. long, attached at one end to bits of sand, earth, or vegetable debris, in water and damp soil.

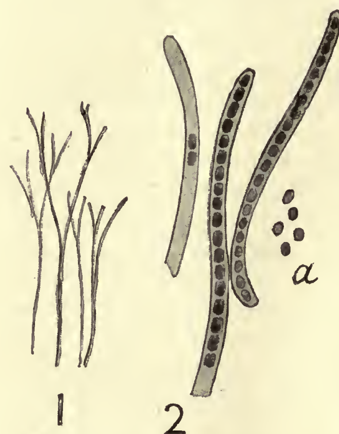


FIG. 28.—*Cladothrix dichotoma*, Cohn.
1. As seen under low magnification (×100).
2. Highly magnified (×750), showing sheath enclosing cells of organism.
a Cells free from sheath.

Each thread consists of a gelatinous sheath, which encloses rod-shaped cells, 3 to 8 μ long and 1 to 2.5 μ broad, arranged end to end throughout its whole length (Fig. 28).

The free ends of the thread appear to be branched dichotomously; this arises not through a true division of the growing point, but is brought about by some of the cells in the sheath dividing and growing out laterally into new filaments.

Some of the cells leave the sheath, becoming flagellated organisms, which swim about for a time; later these settle down and begin to divide and form new threads.

C. dichotoma is not infrequent in water pipes and drains, and one or more closely related kinds are sometimes found in the soil: the "Bismarck brown" *Cladothrix* of Houston, is probably identical with this species.

Gelatine.—The colonies are white, small, from .5 to 2 mm. in diameter, slow in growth, usually with a brown centre, and stain-

ing the surrounding medium a brown colour. The borders have a fringe of filaments round them.

Slight liquefaction of the medium occurs in a week or ten days.

It grows well on gelatine medium (4 to 4.5 per cent. gelatine), to which a very small amount of Lemco has been added; also in dilute bouillon.

Agar.—Whitish colonies, with wrinkled skin-like surfaces; the agar stained brown.

Potato.—Thick grey growth, with brown staining of the potato.

Bouillon.—White flaky colonies, slowly produced in it; the medium is coloured a rich deep brown tint.

Milk.—Slowly made acid; probably peptonized also.

Other species isolated from soil are *Cladothrix rufula*, Wright, which colours media pinkish; *Cl. profundus*, Rav., and *Cl. intestinalis*, Rav., which stain the media brownish; *C. fungiformis*, Rav., which does not stain the medium.

Ex. 70.—Dig up a spadeful of soil from—

1. A sandy field.
2. A field of medium loam.
3. A field of stiff clay.
4. A well manured garden.
5. A peaty heath.

Transfer untouched portions of the soil dug up to sterilized wide-mouthed bottles.

From each of these samples take about 10 grams, and drop them into separate flasks containing 250 c.c. of sterile tap water; shake well.

Then remove 10 c.c. of the turbid water with a sterilized pipette, and add it to another flask containing 250 c.c. of sterile tap water: shake well.

Repeat the dilution once again, *i.e.* 10 c.c. of the latter mixture, into 250 c.c. of water in a third flask.

From this third dilution take 1 c.c. and inoculate an agar plate with it.

Incubate at 22° C., and when the different colonies are well developed examine the organisms of each. Make sketches and describe the colonies.

Try and determine the species by cultivation on gelatine, agar, and potato, and in Bouillon, milk, and other liquid media. (Compare, if necessary, with type cultures, obtainable from the Author.)

4. **Action of Antiseptics and Heat upon the Soil.**—Soil to which carbon disulphide has first been added and then allowed to evaporate is found to be capable of growing a larger crop than a similar untreated soil. The same increased fertility is observed when soil is treated with chloroform, ether, toluene, xylol, and other poisonous volatile compounds. Nitrification and the organisms which produce it are destroyed, but partial sterilization by means of antiseptic agents give rise after a short time to a large increase of ammonium compounds, to which latter the greater fertility of the soil is chiefly due. The ammonium salts may be directly absorbed and assimilated by plants, but partially sterilized soils rapidly become reinfected with living nitrifying bacteria through access of dust, unless special precautions are taken to prevent this from happening, and the crop then receives its nitrogen in the usual manner as nitrate.

Many investigations have been made to determine the nature of the beneficial effect produced by the action of antiseptics upon soil.

It is probable that part of the improvement observed is caused by direct chemical action of those substances upon the insoluble soil constituents, some of the latter becoming available for plant food in the process.

It has been suggested also that these poisonous compounds stimulate absorption by the roots and other physio-

logical functions of the crop, and thus lead to an increased yield, but the fact that the good effect lasts for some time after the compounds have evaporated, militates against the acceptance of this view.

The investigations of Hiltner and Störmer, Pagnoul, Hentze, Russell and Hutchinson show that the beneficial action of antiseptics is connected with alterations in the nature and number of the living organisms of the soil. Hiltner and Störmer showed that the bacterial content was reduced at first by the application of carbon disulphide, but later the numbers increased enormously. Untreated soil, containing 9½ millions of bacteria per gram, contained comparatively few immediately after treatment, but after the lapse of a month or less, the number rose to about 50 millions per gram. They found that the nitrifying and denitrifying organisms were destroyed almost completely, and did not again develop to any extent unless fresh inoculation occurred. Species of *Streptothrix*, which composed about 20 per cent. of the total bacterial flora, were reduced, but the non-liquefying organisms increased very rapidly.

Similar marked increase in numbers of bacteria in partially sterilized soil was observed by Russell and Hutchinson, and found to be correlated with proportional increase in the production of ammonium compounds.

NUMBER OF ORGANISMS PER GRAM OF SOIL.

	AT BEGINNING.	AFTER 9 DAYS.
Untreated Soil .	6,693,000	9,814,000
Toluened Soil .	2,608,000	40,620,000
Heated to 98° C.	393	6,294,100

NITROGEN PRESENT AS AMMONIA. PARTS PER MILLION OF DRY SOIL.

	AT BEGINNING	AFTER 7 DAYS.	AFTER 15 DAYS.
Untreated Soil . .	2.2	1.9	2.7
Toluened Soil . .	4.2	27.9	34.3
Heated to 98° C. .	8.6	27.0	32.6

The total nitrogen-content is not altered, the ammonia being derived from humus, and the bodies of algæ, protozoa, mites, worms, and other living things which have been killed by the antiseptic substance used.

They conclude that in ordinary soils amœbæ and other protozoa devour and keep down the numbers of bacteria; after treatment with toluene or other antiseptics these larger organisms are destroyed, the more resistant bacteria and their spores being then free to develop without competition.

Soil which is heated to 100° C. or over becomes sterile, and when the process is carried out thoroughly, there is, of course, no subsequent development of bacteria unless re-infection occurs. Moreover, heating results in the formation of toxic substances which prevent the germination of seeds and the growth of plants for some time. After the disappearance of the toxic compounds the soil is more fertile than unheated soil of the same kind, the heating process having set free some of its plant food.

Soils which have been partially sterilized by heating to 90° or 95° C. for two or three hours resemble those

which have been treated with antiseptic agents. There is at first a great decrease of bacteria, but this is soon followed by a rapid development of organisms and the production of considerable amounts of ammonium compounds which increase the immediate fertility of the soil. Nitrifying bacteria added to soils which have been heated are checked for a time by the toxic substances produced, whereas they develop freely and at once when added to a soil which has been partially sterilized by treatment with toluene.

CHAPTER IX.

NITRIFICATION.

Nitrification.—When substances such as dung, urine, blood, flesh, or other debris of plants, containing organic nitrogen compounds, are incorporated with the soil putrefaction and decay (p. 100) break them down little by little, the nitrogen sooner or later becoming oxidized and a nitrate is formed. It was by taking advantage of this oxidizing process that nitre or saltpetre (potassium nitrate) was formerly obtained in considerable quantities from so-called "nitre-beds," which were carefully constructed heaps of animal and vegetable refuse, kept moist with urine and other liquids; the nitrogen was oxidized and the potassium nitrate formed was subsequently dissolved out and purified.

This natural production of nitrate, which occurs in varying degrees in most soils, is termed *nitrification.* It was formerly considered a purely chemical process, but in 1877 Schloesing and Müntz, while investigating the chemical changes in sewage, discovered that the oxidation of ammonium compounds into nitrates was the result of the vital activity of living organisms. The process was readily checked by applications of chloroform vapour to the soil, and by heating the latter to a temperature of 100° C. They were not able to isolate the special nitric "ferments," but they determined the

limits of temperature between which the organisms carried on their work, and also the conditions of aeration and other factors which influenced the resulting nitrification. At 50° C. the process is scarcely recognisable, and at 55° C. it is destroyed : the optimum temperature lies near 37° C. Warington, at a later date, showed that nitrification usually takes place in solutions of ammonium salts, with the formation of varying amounts of nitrites and nitrates, and found that in some instances the oxidation of ammonium compounds may proceed as far as the formation of nitrites and there stop, no nitrates being produced. He, moreover, proved that in the nitrification of organic materials, such as milk, urine, and asparagin, the formation of ammonium compounds first takes place.

Although it was found an easy matter to set up nitrification in ammoniacal solutions by the addition of a small amount of garden soil, all the early attempts to isolate and prepare pure cultures of organisms capable of oxidizing ammonium compounds led to unsatisfactory results until 1890, when Frankland and Winogradsky succeeded in the task. They found that the real agents do not grow in bouillon and similar media containing soluble organic substances, and stubbornly refuse to develop on the ordinary gelatine and agar plates. Winogradsky cultivated the organisms in a solution containing ammonium sulphate, potassium phosphate, and a small amount of basic magnesium carbonate. From a medium of this kind devoid of all organic compounds, and undergoing active nitrification, he inoculated plates of gelatinous silica, impregnated with the inorganic salts mentioned, and succeeded in isolating the two kinds of bacteria now known to be the active agents of the nitrification process.

The transformation of complex organic nitrogenous material which occurs in the soils takes place in three stages, namely :—

1. The production of ammonium compounds from the proteins, amino-acids, and other organic bodies present.
2. The oxidation of the ammonium salts thus formed into nitrites.
3. A further oxidation of nitrites into nitrates.

The term nitrification is usually restricted to the two latter stages, that is, to the oxidation of ammonium compounds into nitrates. No organisms are known which will directly nitrify proteins, amino-acids, urea, or similar nitrogenous substances; these must be decomposed first and ammonium compounds prepared from them, the latter being subsequently acted on by the specific nitrifying bacteria.

The preliminary breaking down of the complex organic matter with the formation of ammonium salts is effected by a large number of different kinds of bacteria. Most of the putrefactive group are concerned in the work; bacteria commonly present in soil which are specially active in this direction are *Bs. mycoides*, *Bs. putidus*, *Bs. mesentericus vulgatus*, *Proteus vulgaris*, *Bact. fluorescens liquefaciens*, and *Micrococcus candicans*. Marchal found that *Bs. mycoides* liberated 46 per cent. of the nitrogen of egg-albumen as ammonia, *Proteus vulgaris* 36 per cent., and *Bs. vulgatus* 30 per cent. in twenty days. In addition to bacteria, which are most efficient in neutral or alkaline soils containing little organic matter, certain fungi, such as *Penicillium glaucum*, *Mucor Mucedo*, *M. racemosus*, and species of *Botrytis* and *Torula*, are also responsible for the breaking down

of organic nitrogen compounds, particularly in the acid soils of woods and meadows rich in humus and vegetable debris.

The first step in the oxidation of ammonium salts is brought about by the agency of bacteria, included by Winogradsky in the genus *Nitrosomonas.* They produce nitrites, and pure cultures of them are unable to carry the oxidation further. After being introduced into a nutrient solution of ammonium sulphate (p. 144), an incubation period lasting five or six days occurs, during which little chemical change is effected; afterwards the formation of nitrites goes on rapidly, as much as 90 mg. or more per litre of solution being produced in a day. In the early stages of nitrification of such a solution the nitrite organisms are usually found in a zoogloea state, attached to the particles of magnesium carbonate lying on the bottom of the flask. The individual cells are difficult to observe at first, but after eight or ten days they frequently separate and become motile for a time before forming a zoogloea again. The organisms isolated from western European soils, and known as *Nitrosomonas europæa*, Win., are oval, from 1.2 to 1.8 μ long and .9 to 1 μ thick (Fig. 29), and in a motile state have a single long polar flagellum. The colonies on silica jelly are round, very minute, and at first colourless, highly refractive with a brown edge; later they become dark brown. Several apparently permanent modifications or varieties have been described by Winogradsky; some of them from eastern Europe, Brazil, aud Australia being

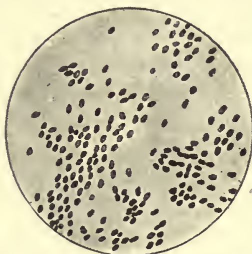


FIG. 29.—*Nitrosomonas europæa*, Win. (×600).

coccus forms 1 to 2 μ in diameter. A very minute variety, .5 μ in diameter, from Java possesses an extremely long flagellum.

Associated with the nitrite organisms in the soil are the nitrate bacteria, which oxidize the nitrites into nitrates and are unable to attack ammonium compounds. They are non-motile rod-shaped bacteria, about 1 μ long, .3 to .4 μ broad, and are included in the genus *Nitrobacter* (Fig. 30); probably several forms exist, but owing to the difficulty of isolating and cultivating them little is known of their morphology. *Nitrobacter* can be grown on nitrite agar, but the colonies are minute and develop very slowly. In suitable media, containing a small amount of sodium nitrite, the formation of nitrates by *Nitrobacter* begins in forty-eight hours or less, but goes on slowly for five or six days, after which oxidation proceeds more rapidly until all the nitrite is completely changed in twelve or fourteen days. In nature both groups of organisms are associated and live together. They are abundant in well and river water, and are distributed through the upper layers of the soil, being most abundant from 4 to 9 inches below the surface, and not usually found at a greater depth than 18 or 20 inches. When both kinds of bacteria are allowed to act upon solutions of ammonium salts the latter are completely changed into nitrites before the formation of nitrates commences, and nitrites can be readily recognized in the solution by the usual tests. Under the conditions which prevail in the soil, however, only nitrates—the end products of

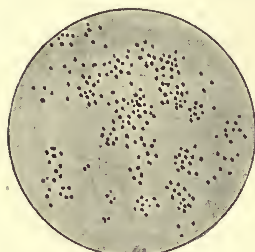


Fig. 30.—*Nitrobacter*, sp. (×600).

their combined action—can be detected, the nitrites which are formed being immediately oxidized as fast as they are produced; the two organisms appear to act symbiotically and simultaneously.

Certain conditions are essential for the nitrification process besides the presence of ammonium salts and the specific nitrifying bacteria. The chief of these are an adequate temperature and suitable degree of moisture, abundant free oxygen and carbon dioxide, a basic substance, such as calcium or magnesium carbonate, darkness, and the absence of soluble organic matter and free ammonia. At freezing point and during winter, when the temperature is below 5° or 6° C., nitrification is checked, but it begins in early spring and continues more and more actively during the warm summer months. At 45° C. the nitrite organisms are destroyed in five minutes, but the nitrate bacteria are not killed below about 55° C.: the optimum temperature for both is near 37° C. Excessive dryness retards nitrification; the best results, according to Coleman's experiments, are obtained in the case of loamy soils, when the latter contain about 16 per cent. of water; when 10 per cent. is present the process is very slow, with 26 per cent. it is stopped or greatly checked. The nitrifying organisms are strongly aerobic, and a good supply of oxygen is essential for the maintenance of their vital functions. On this account the nitrification is most energetic in well-drained, well-aerated porous soils; in clays and water-logged areas the organisms sometimes die out altogether.

Experiments with pure cultures have shown that a basic substance, such as carbonate of sodium, magnesium or calcium, is necessary for the oxidation

of ammonium compounds; in the soil it is usually supplied by calcium carbonate. Where lime is deficient a dressing of chalk or quicklime must be added before the nitrogen present can appear as a nitrate and be made available for the nutrition of crops. Ammonium salts of organic acids undergo as rapid nitrification as ammonium sulphate or chloride when a basic carbonate is available. The necessity for a basic carbonate of the type mentioned, which reacts with ammonium salts to form ammonium carbonate, suggests that the latter is the only compound which can be directly nitrified.

Godlewsky showed that the nitrite bacteria obtain the carbon which which they need for the synthesis of those carbon compounds used in their growth from free carbon dioxide gas; possibly the same holds true for the nitrate forms also. When grown in the presence of insoluble carbonates the organisms do not develop if the atmospheric air supplied to them is first passed through a solution of caustic potash, which absorbs carbon dioxide. It is probable that the carbon dioxide of the air is necessary at first, but later the CO_2 of the carbonate present may be sufficient. They carry on their work best in the dark, bright light being injurious to them. As previously mentioned, soluble organic substances act deleteriously upon both species of nitrifying organisms, the nitrite bacteria being more sensitive in this respect than the nitrate-formers, especially towards nitrogenous substances, such as asparagin or peptone; it is not possible to cultivate either of them in bouillon or solutions of peptone, sugar, and other compounds, which serve well for the growth of most of the ordinary kinds of bacteria. These compounds act upon the nitrifying organisms as phenol, salicylic acid, and other

antiseptics do upon most bacteria, inhibiting or destroying their growth altogether when present in very small quantities. One part in 4000 of glucose or peptone will retard nitrification.

The amounts of various organic substances which were found by Winogradsky and Omeliansky to retard or completely check the two kinds of nitrifying bacteria are given below.—

	Nitrosomonas.		*Nitrobacter.*	
	Percentage amount which		Percentage amount which	
	Retards.	Stops.	Retards.	Stops.
Glucose	.025 to ·05	.2	.05	.2 to .3
Peptone	.025	.2	.8	1.25
Asparagin	.05	·3	.05	·5 to 1
Urea	.2	?	.05	1
Sodium butyrate	·5	1.5	·5	1
Beef broth	10	20 to 40	10	60
Ammonia	—	—	·0005	.015

According to Boullanger and Massol the production of nitrites is checked when the amount of ammonium sulphate in the nutrient medium exceeds 4 or 5 per cent., or when .5 per cent. or less of potassium or sodium nitrite is present; more than 1.5 per cent. of calcium or magnesium nitrite retards it. Almost all nitrites are oxidized to nitrates so long as the amount present does not exceed .05 to .1 per cent., the base having little influence on the process. The development and physiological activity of the nitrate bacteria are diminished in the presence of more than 2 per cent. of nitrite, and also when sodium or potassium nitrate exceeds 2 or 2.5 and calcium nitrate more than about 1.5 per cent. In manure heaps little nitrification occurs owing to the inhibiting action of the excess of organic material present and the want of good aeration.

A very small quantity of free ammonia or of ammonium carbonate, which dissociates easily, also stops the growth and multiplication of the nitrate organisms, but does not much influence the oxidizing power of those already in existence. Any nitrite formed in the soil may be oxidized immediately to nitrate, even in the presence of a certain amount of free ammonia, where species of *Nitrobacter* are abundant. Generally, however, free ammonia is absorbed at once and rendered inert by the constituents of the soil.

The nitrifying organisms are remarkable in possessing the power of manufacturing the organic compounds needed for growth and multiplication of their bodies from simple inorganic salts and carbon dioxide. In this respect they resemble green plants, but they carry on their work without the assistance of chlorophyll or sunlight. The energy necessary for the reduction of carbon dioxide is derived from the oxidation of ammonium compounds; from 33 to 37 grams of nitrogen are oxidized for each gram of carbon assimilated, a fact which to some extent explains the extremely slow development of these organisms.

Every endeavour should be made by agriculturists to assist the nitrifying organisms in their work of preparing available nitrates for the needs of crops. The application of lime, the removal of superfluous water from the soil, and the provision of good aeration by thorough cultivation, especially during summer, are among the most important practices which can be adopted by farmers for the stimulation of these useful soil organisms.

Sulphate of ammonia and manures, which must be nitrified before the nitrogen is available to plants, are best applied to soils of light porous character in which

nitrification is especially energetic, while for soils of damper close texture, in which the activity of the nitrifying organisms are retarded or checked, nitrate of soda or calcium nitrate are more suitable nitrogenous fertilizers.

Ex. 71.—Show the production of ammonia from proteins. Prepare the following solution :—

Di-potassium phosphate	5 gr.
Magnesium sulphate	2.5 gr.
Sodium chloride	2.5 gr.
Water	1 litre.

Take three 200 c.c. flasks and pour into each 100 c.c. of the solution.

Add to one .1 per cent. of gelatine.
„ another .1 per cent. of fibrin.
„ a third .1 per cent. of peptone.

and sterilize.

Inoculate each with *Bs. mycoides* and incubate at 30° C. for ten to fifteen days.

(*a*) Test for ammonia with Nessler's solution.
(*b*) Heat with calcined magnesia and test the gas given off with litmus paper.

Control tubes uninoculated should be tested also.

Ex. 72.—Add to a litre of the inorganic solution in previous experiment.

Glucose	2 gr.
Sodium nitrate	2 gr.

Sterilize; inoculate 100 c.c. of it with *Bs. mycoides*. Incubate at 30° C., and every second day—

(1) Test for nitrites (Ex. 74).
(2) „ ammonia with Nessler's solution.
(3) Find out when the nitrate disappears.

Ex. 73.—To a dilute solution of egg albumin add a dilute

solution of ferrous sulphate to prevent coagulation: sterilize at 100° C. Inoculate with pure cultures of

(*a*) *Bs. mycoides*;
(*b*) *Bact. fluorescens liquefaciens*;
(*c*) *Bact.* (*Proteus*) *vulgare*;

and incubate at 30° C. for ten to fifteen days.

(1) Test for ammonia with Nessler's solution.
(2) Heat with calcined magnesia and test the gases evolved with litmus paper.

Compare with uninoculated sterilized control tubes.

Ex. 74.—Prepare the following solution :—

Ammonium sulphate	2 gr.
Di-potassium phosphate . . .	1 gr.
Sodium chloride	2 gr.
Magnesium sulphate	.5 gr.
Ferrous sulphate	.4 gr.
Distilled water	1 litre.

Pour 50 c.c. into three or four conical flasks and to each add .5 gr. of basic magnesium carbonate. Then add to each about 1 gram of garden soil taken 4 or 5 inches below the surface of the ground, and keep at a temperature of 30° C. Withdraw 5 c.c. from each flask every three or four days and test one-third of each 5 c.c. :—

(1) For ammonia with Nessler's solution.
(2) For nitrites by means of zinc iodide or cadmium iodide starch paste and sulphuric acid.

Dissolve a *small quantity* of starch in water by heating; cool and add a crystal of CdI and heat till dissolved, then add 1 to 2 c.c. of dilute H_2SO_4 ($\frac{1}{10}$ per cent.): a blue colour indicates the presence of a nitrite.

(3) For nitrates after the nitrites have disappeared with diphenylamine and sulphuric acid.

Dissolve a small crystal of diphenylamine in 1 or 2 c.c. of strong H_2SO_4, then add a few drops of the nitrifying solution;

the development of a violet blue colour shows the presence of a nitrate. Since the same colour is given with a nitrite, this test, if intended for detecting nitrates, can only be applied when a previous test for a nitrite has failed.

Note the time—

(1) Taken for the complete disappearance of ammonium reaction.
(2) When nitrite first appears.
(3) When nitrate first appears.
(4) When the nitrite disappears.

Repeat the above experiment, using soil from—

(1) An ordinary arable field.
(2) An old pasture.
(3) A peat bog.
(4) A wood or forest.

Also with soil from—

(1) The surface of the ground.
(2) 6 inches below ground.
(3) 12 inches below ground.
(4) 3 feet " "

Ex. 75.—To test the influence of temperature on nitrification, repeat Ex. 74, keeping some of the flasks—

(1) In a cool place.
(2) At a temperature of 20° C.
(3) At a temperature of 30° to 35° C.
(4) Add to one of the flasks soil which has been heated to a temperature of 100° C. for half an hour.

Ex. 76.—To test the inhibiting effect of soluble organic compounds on nitrification, repeat Ex. 74, adding to a series of flasks .01, .03, .3, 1, and 3 per cent. of—

(1) Dextrose,
(2) Peptone,
(3) Urea

respectively.

Ex. 77.—Take six or eight conical flasks with bases about 4 or 5 inches in diameter: pour into each 50 c.c. of the solution given in Ex. 74, and add .5 gr. of basic magnesium carbonate. Inoculate one of the flasks with about 1 gram of

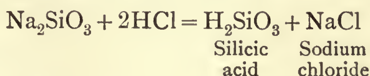
garden soil, and keep at a temperature of 30° C. Test a small amount of the solution every fourth day with Nessler's solution until no reaction occurs. Then add 1 c.c. of a 10 per cent. solution of ammonium sulphate and allow this to nitrify, testing every three or four days until the ammonium salt is completely changed. Then transfer some of this nitrifying solution by means of a platinum loop to a second flask. When the solution in the latter gives no reaction with Nessler transfer some of it to a third flask, and similarly some of the third to the fourth, and so on. By cultivating several generations in a medium devoid of organic matter, in this way it is possible to eliminate most of the non-nitrifying bacteria present in the soil and obtain fairly pure cultures of the nitrifying organisms.

Try and obtain a pure culture which will produce nitrites or nitrates when introduced into fresh ammoniacal solution, and yet give no growth or turbidity when a drop is transferred to bouillon tubes and incubated at 30° C.

Ex. 78.—To obtain pure cultures of the nitrifying organisms their isolation must be carried out on "silica jelly" plates.

(*a*) For the preparation of "silica jelly" Winogradsky and Omeliansky recommend the addition of a solution of pure "water glass" (sp. gr. 1.05 to 1.06) to an equal quantity of hydrochloric acid (sp. gr. 1.1).

Pour the solution of sodium silicate slowly into the dilute hydrochloric acid (not the acid into the silicate solution), shaking the mixture gently. The following reaction takes place, and a solution of silicic acid is obtained, mixed with sodium chloride:—



$$Na_2SiO_3 + 2HCl = H_2SiO_3 + NaCl$$

Silicic acid — Sodium chloride

To get rid of the sodium chloride pour the mixture into a parchment dialyser and float or suspend the latter in distilled water; the sodium chloride diffuses through the parchment into the water, which should he changed twice a day: the silicic acid is a colloidal gelatinous body and does not pass through, but

remains in the dialyser. (Great care must be taken to procure a sound piece of parchment or dialyzing membrane, or it will not be possible to obtain a solution of silicic acid of the right strength to form a firm solid jelly.) Test small portions of the solution taken from the dialyzer with silver nitrate from time to time, and when no milkiness is given with this reagent the solution may be poured into a stoppered flask and sterilized: it does not keep well and is best used in a day or two, since it is liable to lose its clearness and become unfit for use.

(*b*) When required for the preparation of "silica jelly" plates, for the cultivation of the nitrite organism, the silicic acid obtained by the above method is mixed with the following solution, which must be sterilized:—

Ammonium sulphate	.3 gr.
Di-potassium phosphate . . .	.1 gr.
Sodium chloride	.2 gr.
Magnesium sulphate	.05 gr.
Ferrous sulphate	.04 gr.
Water (twice distilled)	100 c.c.

To 50 c.c. of the silicic acid solution add 2.5 c.c. of the above and enough of a sterilized mixture of fine magnesium carbonate and water to make the whole milky.

(*c*) (i) Inoculate as rapidly as possible with a loopful of the purest culture obtained by the successive cultivation of the nitrifying organism, as in Ex. 77, and after shaking gently pour at once into a sterilized Petri dish and allow the liquid to solidify; incubate at 30° C., or

(ii) Pour the mixed silicic acid and ammoniacal solution into a Petri dish, and after the "jelly" has "set" place a drop of inoculating medium on the surface and very gently spread it in streaks with a sterilized bent glass rod, taking care not to tear the surface of the jelly.

As the silicic acid solution gelatinizes in a short time when mixed with the ammonium sulphate solution, the mixing and inoculation must be carried out rapidly. Incubate at 30° C.,

placing the dish cover downwards so that the exuded water will not remain on the surface of jelly. The deep colonies of the nitrate organisms are very small and appear as refractive points with dark rims, and as a rule gradually become surrounded by a small clear space, from which the magnesium carbonate is dissolved as growth and nitrification proceeds: examined with a low-power microscope they appear granular. Remove colonies or small portions with a fine capillary tube, obtained by melting a piece of ordinary thin glass tube and drawing it out rapidly when soft; transfer to flasks containing the ammonium sulphate solution (Ex. 74). The tip of the tube conveying the nitrite organism can be easily broken off and left in the flask by pushing against the bottom of the latter. Incubate the inoculated solution at 30° C. and

(1) test for nitrate at intervals;
(2) examine it with the microscope and note the form, size, and motility of the organisms present; stain some;
(3) test for the presence of foreign, non-nitrifying species by introducing some of the solution into bouillon: note if the latter remains clear and without signs of growth for twelve to fourteen days at least.

The nitrite organisms stain with carbol-fuchsin and are roundish or oval, 1.25 to 1.8 μ long and .9 to 1 μ thick, somewhat irregular in size.

Ex. 79.—*Stevens-Temple Silica Jelly.*—The following method of preparing a "silica jelly" is due to Stevens and Temple, and has the advantage of avoiding the process of dialysis, which in the hands of beginners is troublesome and uncertain in its results. The method leaves a certain amount of sodium chloride in the "jelly," but this has not been found to interfere with the cultivation of nitrifying organisms.

(*a*) Take 3 to 4 c.c. of a solution of sodium silicate (water glass). Place in a porcelain dish and add 5 to 10 c.c. of concentrated hydrochloric acid to precipitate the silicic acid. Evaporate to dryness: moisten again with hydrochloric acid

and evaporate to dryness a second time. Then wash and transfer to a Swedish filter paper, the weight of whose ash is known. Wash the precipitate until the water gives no cloudiness with silver nitrate solution. Burn the filter with the precipitate upon it in a weighed platinum or porcelain crucible and heat to redness. Allow to cool, and weigh the silicic anhydride (SiO_2). Calculate the amount of silicic anhydride in 1 c.c. of the original solution.

(*b*) Dilute the amount of water glass which you wish to use for preparation of the jelly until the solution contains 4 or 5 per cent. of silicic anhydride. Prepare hydrochloric acid of such strength that 1 c.c. just neutralizes 1 c.c. of the "water glass" solution, using methyl orange as an indicator. Take 104 c.c. of the hydrochloric acid and add to it 100 c.c. of the "water glass" solution: the excess of acid prevents coagulation during the subsequent sterilization. The solution should be poured into tubes or flasks and sterilized at 120° C. for fifteen minutes, or at 100° C. for twenty minutes, on three successive days: 10 to 20 c.c. are convenient amounts to place in tubes.

Since the medium cannot be liquefied after once it has "set," tubes for "slants" must be put in position as soon as possible after mixing.

(*c*) Inoculate the tubes from the flasks containing nitrifying organisms (Ex. 77) and add to each 10 c.c. of sterilized solution, .5 c.c. of the ammonium sulphate solution (in Ex. 74) and a drop or two of sodium carbonate solution, enough to make the whole alkaline. Pour into Petri dish at once and allow to solidify.

(*d*) Inoculation may be made on the surface of the solidified medium as in Ex. 78 (*c.* ii).

Ex. 80.—Isolate and grow the nitrate organism. Prepare the following solution:—

Sodium nitrite (puriss)	1 gr.
,, carbonate (calcined) . . .	1 gr.
,, chloride	.5 gr.

Di-potassium phosphate (K_2HPO_4).	.5 gr.
Ferrous sulphate	.4 gr.
Magnesium sulphate	.3 gr.
Distilled water	1 litre.

Take six or eight conical flasks with wide bases, 4 to 5 inches in diameter: pour into each 50 c.c. of the solution, and add .5 gram of basic magnesium carbonate. Inoculate one of the flasks with about 1 gram of garden soil and keep at a temperature of 30° C. Test the solution for nitrites every two or three days with zinc or cadmium iodide starch paste, *and when this reaction ceases* test for nitrates with diphenylamine and sulphuric acid (Ex. 74). Then add 1 c.c. of a 10 per cent. solution of sodium nitrite and test again similarly until the added nitrite disappears. Transfer a loopful from this flask into a second flask of the solution, and when nitrate is formed in the second transfer again to a third, and from the third to a fourth.

The nitrate organisms may be grown on silica jelly impregnated with the above nitrite solution and magnesium carbonate, in the same manner as that described for the nitrite bacteria, Ex. 78.

They grow, however, on the following agar medium, which is more easily prepared :—

Sodium nitrite (puriss)	2 gr.
,, carbonate (calcined)	1 gr.
Di-potassium phosphate	.2 gr.
Agar	15 gr.
Tap water	1 litre.

From the last of several successive generations of cultivations in the nitrite solution, already given above, inoculate—

(i) The melted agar medium in the usual manner with a loopful of the solution: pour into a Petri dish, turn it upside down in order to allow exuded water to run off the surface of the medium.

(ii) Make a streak culture on solidified nitrite agar in a Petri dish. Incubate for three or four weeks at 30° C. The surface colonies

look like very small watery drops with granular contents: the deep ones are exceedingly small, brown, kidney or spindle shaped. The larger colonies observed are generally impurities. Transfer some of the smallest colonies by means of capillary tube (Ex. 78 *c.* ii) to flasks of the nitrite solution, using low-power microscope for selection of the colonies, and

(1) test for disappearance of nitrite and appearance of nitrate;

(2) examine a drop of the solution and note the form and size of organisms observed;

(3) inoculate ordinary slightly alkaline bouillon and note if turbidity and growth occurs; carry on the incubation at 30° C. for two weeks at least.

From cultures which are bouillon sterile the organisms may be obtained and stained with carbol fuchsin: they are usually rod-shaped, about 1 μ long and .3 to .4 μ thick, or in some instances somewhat oval and 1 to 1.5 μ long and up to .8 μ thick.

CHAPTER X.

DENITRIFICATION.

Denitrification and Reduction of Nitrates and Nitrites.—In the process of nitrification ammonia or its compounds are first oxidized to a nitrite, and finally to a nitrate by certain specific organisms, as already described in the previous chapter. Many bacteria are known which carry on processes of reduction the reverse of this, oxygen being removed from nitrates to a greater or lesser extent. The reduction may be of several kinds, namely :—

1. The nitrates may be reduced to nitrites and no further.
2. The oxygen may be taken from nitrates and nitrites, with the ultimate formation of ammonia.
3. Nitrates and nitrites are sometimes reduced with the evolution of nitric (NO) and nitrous oxides (N_2O).
4. The nitrates and nitrites may be reduced with the final production of free nitrogen.

Although the term *denitrification* has been sometimes loosely employed to denote all these forms of reduction, it is now more strictly applied to the decompositions of nitrates and nitrites, which result in the evolution of free

nitrogen. The ability to reduce nitrates to nitrites is possessed by a large number of different kinds of bacteria: out of 32 forms isolated by Frankland from air and water more than half of them had this property, and of 109 examined by Maassen about 90 showed the same power. Many of these, and others equally commonly distributed, are apparently able to carry the reduction further and give rise to ammonia; the latter, however, may be derived from proteins and other nitrogenous organic compounds often present where bacterial action is going on. It is in cases where the nitrates are decomposed with the formation of the gaseous oxides or free nitrogen that the process becomes of special interest to the agriculturist, since these changes involve a loss of valuable fertilizing material from the soil or from manure heaps. The conditions essential for denitrification are:—

a. The presence of certain species of bacteria.

b. A supply of nitrates.

c. A considerable amount of easily assimilated organic substances.

d. Total absence or a very limited access of free oxygen.

These, it will be noticed, are practically opposite conditions to those required for nitrification. The exclusion of any one of them checks denitrification. A large number of kinds of bacteria have been isolated which are capable of decomposing nitrates with the evolution of free nitrogen. Many of them are found in water and in the soil. They are also especially abundant upon straw and in the dung of the horse, cow, and other herbivorous animals; in fæces of man and carnivorous animals they are rarely present. All these organisms are aerobic

species and grow well enough in bouillon when supplied with free oxygen, but do not reduce nitrates to more than a slight extent under such circumstances. When cultivated anaerobically or with a diminished supply of air they take oxygen from nitrates or nitrites, using it for the respiratory combustion of comparatively large amounts of organic carbon compounds. That the want of oxygen is the chief cause of denitrification may be deduced from the cultivation of *Bact. pyocyaneum*, or a similar organism with and without nitrite in the presence and absence of air respectively. Growth and multiplication take place in alkaline nitrite bouillon under aerobic conditions without decomposition of the nitrite, but when cultivated as an anaerobe the nitrite disappears rapidly with the evolution of free nitrogen. Without the nitrite under anaerobic conditions little or no growth occurs, and respiration is stopped even when suitable oxidizable material is available. The power of utilizing organic compounds apparently as the source of energy for the reduction of nitrates which takes place in the denitrification process, varies considerably with different species of bacteria. Some of these can oxidize certain carbohydrates and acids which are not attacked by other species. For instance, Salzmann found that *Bact. Hartlebii* made use of xylan, glucose, lactose, and maltose, but none of these were of service for the growth of *Bact. Stutzeri.*

The amount of nitrate decomposed is proportional to the amount of easily assimilated carbon compounds available. Those substances which serve best for the denitrifying species of bacteria are neutral salts of organic acids, especially butyric, lactic, and citric acids; acetic, propionic, valerianic, and other acids are

useful also. Mixtures of these with glucose, starch, and other carbohydrates are utilized also, but not usually the latter alone. Many of the alcohols, such as ethyl, propyl, and isobutyl alcohols and glycerine are oxidized by them, and hippuric and uric acids are utilized also by some species. Many organic compounds, which are not found to be of service to pure cultures of denitrifying bacteria, are indirectly utilized when the latter are associated with putrefactive organisms. A number of the compounds so readily utilized by denitrifiers are produced by the action of putrefactive organisms upon the complex insoluble compounds in animal and vegetable tissues, humus and the undigested materials in fæces. Some organisms, such as *Bact. denitrificans*, L. and N., are known which can set free nitrogen from nitrites; they are unable to attack nitrates directly, but when grown symbiotically or along with *B. coli* and other species which are able to carry out for them the preliminary reduction of the nitrate to nitrite, free nitrogen is evolved. In manure and compost heaps there is little or no denitrification with its consequent loss of nitrogen in the gaseous form, since the necessary nitrates are generally missing, the excess of soluble organic matter and absence of aeration preventing their formation except in the upper layers of the heaps. In well-aerated composts, however, and in farmyard manure loosely put together nitrification may occur and be followed by denitrification when the heaps become saturated with water through exposure to heavy rains. In ordinary arable soils loss of nitrogen by denitrification is uncommon, for where nitrates are abundant the easily assimilated carbon compounds upon which denitrifiers depend are wanting. That it may occur in poorly aerated, water-logged soils under anaerobic

conditions, was shown in an experiment carried out by Warington. A column of soil was saturated so as to drive out the air from the small spaces within it, the water being allowed to percolate through so as to extract all the nitrate present. After no reaction for nitrate was obtained, a certain amount of nitrate of soda was then placed on the surface of the soil and watered into it daily. It was found that only 21 per cent. of the amount applied to the soil could be recovered, 79 per cent. being lost by denitrification: transverse fissures developed across the soil at some depth below the surface, these being due to the èvolution of nitrogen and carbon dioxide gas.

In 1897 Wagner and Maercker drew attention to some remarkable results obtained from the growth of oats, mustard, and other plants in pots, to the soil of which fresh undecomposed farmyard manure had been applied alone or along with nitrate of soda. They found that where fresh horse or cow dung were added in large amount the crop yield was less than where no manure at all was used, and also discovered that under certain conditions the application of dung greatly depressed the produce from soil which had received nitrate of soda as well. The following are typical results:—

OATS (*Maercker*).

	Yield of Grain and Straw.
Soil without manure . .	44.82 grams.
" with nitrate of soda (1.5 grams N.) . .	128.37 " (186°/。 increase).
" with horse dung (2.25 grams N.)	35.91 (1) " (20°/。 decrease).
	23.33 (2) " (48°/。 decrease).

MUSTARD (*Wagner*).

	Yield of Grain and Straw.
Soil without manure . .	1.6 grams.
„ with nitrate of soda (2 grams N.) . .	36.6 „
„ with horse dung (2 grams N.)	.5 „
„ with cow dung („)	.4 „

The amount of nitrogen recovered in the crop when nitrate of soda was used alone in one case was 77 per cent., whereas only 52 per cent. was recovered when the nitrate was applied in conjunction with horse dung. That the difference was due to the destructive action of the horse dung on the nitrate was concluded from a determination of the nitrates extracted with water from soil treated with nitrate and nitrate plus dung in cases where no crop was grown. A pot of soil to which .5 gram of nitrate of soda was added was compared with one to which dung and the same amount of nitrate were applied and left for an interval of six weeks. While 93 per cent. of the nitrate in the former appeared in the extract, the pot receiving the dung in addition yielded only 42 per cent.; moreover, the filtrate from soil to which no addition of nitrogenous manure had been made was richer in nitrate than that to which horse dung had been applied, the latter having not only destroyed the added nitrate, but also that naturally present in the soil as well.

The addition of chopped straw in large quantities to soil containing nitrate has a similar effect to that produced by fresh dung.

The correct interpretation of the results of Wagner's and Maercker's work is of importance since casual consideration might lead to the conclusion that fresh horse

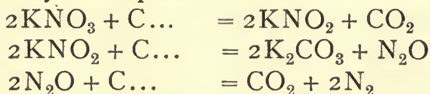
and cow manure are practically valueless for the nutrition of plants, and that their addition to the soil is dangerous on account of the consequent destruction of nitrates which follows such practice. It must be noted that in all the experiments very large amounts of fresh dung were used, far in excess of what would be used in farm practice. Wagner attributed the bad effect of dung and straw chiefly to the large numbers of denitrifying bacteria which they contain. No doubt these materials add denitrifying bacteria to the soil, but the loss of nitrogen observed is due more particularly to the large quantity of easily assimilated organic matter which the dung contains, for without this material denitrification cannot proceed. Well rotted dung or farmyard manure which has been ploughed into the land some time is found to have little or no destructive effect upon nitrates added subsequently, and does not reduce the yield of crops in the same way as fresh dung does: the numbers of denitrifying organisms are not reduced by rotting of the manure, but the easily oxidized organic matter which they need for their special denitrifying effect is greatly diminished by the putrefactive changes and decay which takes place in an aerated heap or in the soil.

The reduction of crop under the conditions of these pot experiments, where large amounts of fresh dung or straw are used, is due in part to the destruction of nitrates and loss of gaseous nitrogen ; but some of it is due to another cause, namely, the assimilation of nitrates by certain species of bacteria added by the dung and straw, or stimulated by these materials into active growth, and there occurs a locking up of nitrogen in an organic form in the bodies of these organisms. Berthelot and others have shown that the organic

nitrogen compounds of the soil may be increased at the expense of the nitrate in it. Such syntheses of insoluble nitrogenous material temporarily reduce the amount of nitrogen available for the growth of ordinary field crops: after death of the bacteria, however, nitrification sets in, and the nitrogen becomes again of service for the nutrition of green plants. Experience of farmers and experiments at Rothamsted and elsewhere have shown that under ordinary conditions of farming a good return is generally obtained from the nitrogen of nitrate of soda, even when applied with dung, so long as the latter is used in moderate amounts. It is only in gardens where 50 to 100 tons of fresh dung per acre are occasionally employed that denitrifying effects such as those observed in Wagner and Maercker's pot experiments are likely to be encountered.

A large number of species of bacteria are known to possess the power of reducing nitrates to nitrites; a much smaller number, however, are found capable of destroying nitrates with the evolution of free nitrogen.

From the recent investigations of Beijerinck and Minkman, it would appear that the denitrification of a nitrate in the soil usually results in the formation of considerable amounts of carbon dioxide and nitrous oxide gases, from the latter of which free nitrogen is ultimately evolved. The decompositions occurring in the process may be expressed thus:—



$$2KNO_3 + C\ldots = 2KNO_2 + CO_2$$
$$2KNO_2 + C\ldots = 2K_2CO_3 + N_2O$$
$$2N_2O + C\ldots = CO_2 + 2N_2$$

where C... represents an organic carbon compound.

They found that in some instances the mixed gases given off from bouillon with 5 to 12 per cent. of nitrate

in it contained as much as 80 per cent. of nitrous oxide.

Many denitrifying bacteria, such as the forms examined by Gayon and Dupetit, Warington, Giltay, and Aberson, are insufficiently known to warrant inclusion here; the chief of the forms capable of setting free nitrogen which have been isolated and accurately described are mentioned below:—

Bact. Stutzeri, L. and N. (*Bact. denitrificans II.*, Burri and Stutzer).—A feebly motile organism isolated from straw, soil, and the air; grows best with small supply of oxygen. Generally rods with rounded ends and non-motile, grown on agar, 1.5 to 2.5 μ long, 0.5 to 1 μ thick. Sometimes spindle shaped. In nitrate bouillon the rods are thinner (.25 to .3 μ in thickness). Stains more at poles than in middle, except in very young cultures.

Gelatine.—Not liquefied. Surface colonies dull white, 1 to 2 mm. diameter, with characteristic radial rib-like markings and raised rims: deep colonies are small, white, round or spindle form with ragged edge. Stab beaded, white, slimy after a time.

Agar.—Surface colonies somewhat similar to those of gelatine at first; later they become flatter and less distinct in character.

Potato.—Growth ribbed; pale flesh colour on potato feebly alkalinized with .05 per cent. sodium carbonate.

Nitrate bouillon (.3 per cent.).—Denitrification in one to two days or less, with abundant frothing and sediment.

Bact. denitrificans, L. and N. (*Bac. denitrificans I.*, Burri and Stutzer).—An aerobic species obtained from horse dung, straw, and soil. On agar 1.5 to 2.5 μ long, .75 μ thick; in bouillon 2 to 3 μ long. Rods mostly non-motile, stains uniformly with methyl-violet, but more at poles with carbol-fuchsin.

Gelatine.—Surface colonies are not usually found to develop in

spite of the aerobic character of the organism. When present are white, somewhat transparent and slimy later, with ragged edge. Deep colonies small, rounded, and yellowish white. Stab threadlike, very delicate growth all over surface.

Agar.—Surface colonies are produced on agar; roundish edge; they are very thin often, with a whitish centre, and from .5 to 3 cm. across.

Potato.—On alkalinized potato (.05 per cent. Na_2CO_3) little growth at room temperature: at 30° C. in two days, dirty brownish red: with shining surface.

Nitrate Bouillon.—Denitrification of nitrate only in presence of *B. coli* or other reducing organism. Denitrifies a *nitrite* bouillon alone, with frothing.

Bact. agile, Amp. and Gar.—A facultative anaerobic organism isolated from cow dung; very short, motile rods, 1 to 1.5 μ long, .1 to .3 μ thick.

Gelatine.—Not liquefied. Surface colonies, round, white, scarcely visible (150 to 170 μ diameter), and grow slowly. In nitrate gelatine small gas bubbles surround them. Stab thin and slightly beaded, with small growth at surface, 1 mm. diameter, greyish.

Agar.—White, slimy colonies, which remain small.

Potato.—On alkalinized (.05 per cent. Na_2CO_3) potato, growth is very slow, thin, yellow or dirty in brown colour.

Nitrate Bouillon.—Grows well in this medium, denitrifying and producing froth with fine gas bubbles and turbidity.

Bact. Hartlebii, Jensen.—A motile organism isolated from soil. Rods, 2 to 4 μ long, .75 μ thick; stains easily and generally uniformly.

Gelatine.—Not liquefied. Surface colonies very small, 1 to 3 mm. in diameter, slimy, semi-transparent and white, usually with entire border. Stab beaded, with a white slimy growth at the surface, 3 to 6 mm. diameter.

Agar.—Surface colonies slimy, translucent; later more watery; deep colonies small white and entire.

Nitrate Bouillon.—Turbid in twenty-four hours; denitrification and frothing in two to three days.

Bact. centropunctatum, Jensen.—Isolated from cow dung and fæces of guinea-pig. On agar under aerobic conditions is coccus-like, .3 to .5 μ in diameter, with a large slimy capsule 3 μ diameter. In nitrate bouillon is rod-shaped, motile, and like *Bact. Stutzeri.* Under anaerobic cultivation are large, oval organisms 5 μ long, 1 μ thick, which stain chiefly at the ends. On account of thick capsule is difficult to stain unless washed in xylol and alcohol first on cover slip.

Gelatine.—Not liquefied. Surface colonies thin, whitish translucent, ragged borders; deep colonies, small white entire border. Stab beaded, with white spreading growth on surface.

Agar.—Surface colonies thick, moist, not slimy, dirty grey in colour.

Nitrate Bouillon.—Turbid in one to two days, with frothing and denitrification.

Bact. Schirokikhi, Jensen.—A feebly, motile, aerobic organism, isolated from horse dung. Rods with rounded ends, usually three or four together in a chain when grown in bouillon; in pairs on solid media. Spores are abundant, and produced in two days.

Gelatine.—Liquefied to a pale bluish liquid in which are yellow grains. Surface colonies are slow in developing; on the third day, at 20° C., are about 1 to 2 mm. in diameter, in five days or less colonies run together. Stab; rapid, funnel-shaped liquefaction, liquid pale grey at first, yellowish afterwards, with sediment.

Agar.—Surface colonies develop sooner than on gelatine, and are star-shaped in twenty-four hours at 37° C., with a raised centre surrounded by pale blue rays. In three or four days the colonies become rounded, 2 to 4 cm. in diameter, with a yellowish brown zone round each.

Potato.—On alkalinized potato (.05 Na_2CO_3) growth is light brown.

Nitrate Bouillon.—(25 per cent. KNO_3). Denitrification complete in five to eight days, at 30° to 35° C.

Bact. filefaciens, Jensen.—A non-motile organism isolated from an old impure culture of *Bact. Stutzeri.* Rods usually .5 to 1.5 μ long and .5 to .75 thick; older cells stain irregularly.

Gelatine.—Not liquefied. Surface colonies thin, dull and somewhat slimy; deep colonies very small, white, round. Stab beaded, with well developed white surface growth.

Agar.—Surface colonies dirty white, slimy, can be drawn out into long strings.

Nitrate Bouillon.—Denitrification in one to two days at 30° C.; liquid pretty clear with a stringy sediment.

Bact. nitrovorum, Jensen.—A non-motile organism isolated from horse dung. Rods very small, .5 to 1 μ long, .5 μ thick.

Gelatine.—Not liquefied. Surface colonies very small, .2 to 5 mm. in diameter, white, slimy, and round; deep colonies scarcely visible. Stab slender and beaded, its surface growth 6 to 10 mm. diameter, with concentric rings sometimes.

Agar.—Surface colonies, dirty, white, slimy, 2 to 4 mm. in diameter.

Nitrate Bouillon.—Slightly turbid in twenty-four hours; denitrification in two or three days, with little froth; sand-like sediment.

Vibrio denitrificans, Sew.—A peculiar variable bacterium isolated by Sewerin from horse dung. In bouillon comma and spirillum forms occur as well as rods 1 to 10 μ long; branched and triangular involution forms are also found after four or five days. On solid media usually rods 2 to 4 μ long, .5 μ thick.

Gelatine.—Not liquefied. Small, round, white surface colonies, slow in development. Stab beaded.

Agar.—In two days at 30° C. small (1 to 2 mm.), bluish-white, slimy colonies.

Nitrate Bouillon.—Denitrification occurs in two or three days with turbidity and only a slight frothing.

Vibrio denitrificans, Höflich.—A motile vibrio isolated from horse and cow dung, straw and soil. Both rods and vibrio forms

occur, the latter 1.4 to 2.3 μ long, .4 to 5 μ thick, often many together in zoogloea.

Gelatine.—Not liquefied. Surface colonies slightly raised, whitish, smooth at first, later puckered, and 2 to 3 mm. in diameter; deep colonies, small (1 to 2 mm.), bluish-white, blackberry form, with opaque yellowish brown centre.

Agar.—Surface colonies, rounded in forty-eight hours at 35° C., 1 to 2 mm. diameter, centre milk-white, with whitish cleaner space round.

Potato.—At 35° C. in two to three days, a yellowish brown, slightly raised, growth not spreading much.

Nitrate Bouillon (3 per cent.).—In twelve hours turbid; in twenty-four to thirty-six hours much froth, with complete denitrification and little sediment; remains turbid after frothing ceases. Differs from the preceding in form of deep colony, more uniform staining, want of sliminess, and abundant frothing in nitrate bouillon.

Bacillus (Proteus) denitrificans, Höflich.—An aerobic "proteus-like" bacterium, isolated from horse and cow dung, straw and soil. In bouillon the organisms are motile, 5 to 10 μ long and .3 to .4 μ thick; they grow into long threads (100 μ long or more), which often break up in three or four days into rods, vibrio, and even coccus forms, all without spores.

Gelatine.—Not liquefied; the surface colonies are round or irregular at first, and gradually extend over 2 to 3 sq. cm. of surface as a filmy growth, bluish-white and semi-transparent. Deep colonies are whitish, and remain small. Stab a delicate whitish line of very small colonies.

Agar.—In twenty hours, at 35° C., the surface colonies are bluish-white, transparent, very thin, 1 cm. in diameter. They spread very rapidly, and cover the plate in about two days.

Potato.—No visible growth.

Nitrate Bouillon (3 per cent.).—In twenty hours, at 35° C., diffuse turbidity with denitrification and formation of froth and

fine bubbles. Later liquid clears and froth disappears, leaving faint iridescent scum and whitish light sediment.

Ex. 81.—Prepare the following media:—

1. *Nitrate Bouillon.*

(1)

Extract of lean beef (see p. 56) . .	1 litre.
Peptone	10 gr.
Sodium chloride	5 gr.
„ nitrate	3 gr.

(2)

Lemco's Extract	5 gr.
Peptone	10 gr.
Sodium nitrate	3 gr.
Water	1 litre.

No. 1 usually gives best results. Make both just neutral with sodium carbonate and sterilize as usual.

2. *Nitrate Gelatine.*

Nitrate bouillon as above . . .	1 litre.
Gelatine	100 gr.

Neutralize with sodium carbonate; place 10 to 15 c.c. in tubes and sterilize.

3. *Nirate Agar.*

Nitrate bouillon as above . . .	1 litre.
Agar-agar	15 gr.

Neutralize as above, tube and sterilize.

Ex. 82.—(1) Add 5 grams of fresh horse dung to—

Water	100 gr.
Sodium nitrate	.3 gr.

Incubate about 30° C.: notice frothing, and test for nitrate with diphenylamine and sulphuric acid, as in Ex. 74, every two days for a fortnight. Nitrate usually disappears in six or eight days.

(2) Repeat above, using 5 grams of chopped straw instead of dung.

(3) Boil the solution after adding the dung; incubate and test for nitrate as before. Does boiling check denitrification?

Ex. 83.—Isolate denitrifying organisms from dung, soil, ditch water, and straw.

(*a*) Inoculate tubes containing 10 to 15 c.c. of neutral nitrate bouillon (Ex. 81) with small pieces of fresh dung, soil, or other material to be examined, or with watery extracts of them. Incubate at 30° to 35° C. and when frothing is well developed inoculate ordinary agar and gelatine plates from the fermenting medium. Transfer small portions of the colonies which appear on the plates into tubes of nitrate boullion; observe frothing and test for disappearance of nitrate with diphenylamine (Ex. 74) during a fortnight, or longer if necessary.

It is generally better to incubate the medium inoculated directly with the dung or its extract anaerobically in a yeast flask at 35° C. (Ex. 140), as this checks the development of aerobic, liquefying, and non-denitrifying species and makes the subsequent isolation of denitrifying species less laborious.

(*b*) Similar results are obtained thus: Shake up small pieces of dung, soil, etc., in sterilized water and inoculate agar and gelatine plates with a drop of this extract in the usual way. Test the denitrifying power of the several colonies obtained by transference to nitrate bouillon as before.

Ex. 84.—Take a glass cylinder 2 feet long, 2 or 3 inches in diameter, and close one end with a cork through which a small piece of glass tube is passed. Place in an upright position and half fill the cylinder with garden soil closely packed, first covering the cork on the inside with coarse calico so that the soil does not pass through the small glass tube: close the lower end of the latter with a cork. Now pour water into the wide open end of the cylinder and wait until the soil is saturated; then add enough to fill the empty space above the surface of the soil. Remove the cork from the small glass tube and allow the water to percolate through: test for nitrates with diphenylamine (Ex. 74) every second day. The water which drains through

should be poured back again on the soil so as to keep the latter saturated the whole time. The nitrates are ultimately destroyed by denitrification, when the water coming through shows no reaction with diphenylamine.

Ex. 85.—Inoculate tubes of nitrate bouillon (Ex. 81) with a gram of soil or fresh horse or cow dung, and

(1) Incubate at room temperature as an aerobe.
(2) „ 35° C. „ „
(3) „ 35° C. „ anaerobe in yeast flask.

Note (*a*) time taken to develop froth; (*b*) presence or absence of turbidity and sediment; (*c*) disappearance of nitrate (diphenylamine test).

Ex. 86.—Melt some tubes of nitrate gelatine (Ex. 81) in warm water, and introduce into each a loopful of nitrate bouillon containing any denitrifying species isolated in Ex. 83. Gently shake the tubes to distribute the bacteria in the medium: allow the latter to solidify and incubate at 18° to 20° C. Note the development of gas bubbles in the gelatine (Fig. 31).

Fig. 31.—Gas bubbles developed in nitrate gelatine by denitrifying organism.

Ex. 87.—Prepare the following solution:—

Calcium tartrate . .	10 gr.
Potassium nitrate . .	10 gr.
Di-potassium phosphate .	.25 gr.
Tap water . . .	500 c.c.

Sterilize.

Fill several 50 c.c. sterilized flasks to within an inch of the top with the solution, and introduce into each 1 to 2 grams of soil, dung, or other material. Then completely fill to the brim and close with a cork, through which is passed a small piece of glass tube; some of the solution will run out of the tube, but the flask will be full: inoculate at 28° C. Denitrification soon begins, and the gases (CO_2 and N) evolved drive out some of

the solution, but the space in the flask is filled with inert gases and the culture grows under practically anaerobic conditions. From this flask introduce some of the fermenting liquid into another filled completely in the same manner with the culture solution. When frothing has gone on some time in the second flask inoculate a third from it. In this way a culture can be obtained containing denitrifying species almost entirely, the aerobic non-denitrifying species present in the soil or dung being suppressed. Inoculate agar and gelatine plates from the last flask, and isolate and examine the organisms which develop.

Ex. 88.—A good medium in which to grow denitrifying species is :—

Giltay's solution.

Sodium nitrate	2 gr.
Citric acid	5 gr.
Magnesium sulphate	2 gr.
Di-potassium phosphate . . .	2 gr.
Calcium chloride	.2 gr.
Water	1 litre.

Neutralize with a solution of sodium carbonate, and add a drop of ferric chloride.

Cultivate denitrifiers in flasks or tubes of this solution, and note increasing alkalinity as growth proceeds for three or four weeks. Oxygen removed from the sodium nitrate gives rise to caustic soda (NaOH), some of which unites with the CO_2 produced from the oxidation of the citric acid, and forms sodium carbonate (Na_2CO_3). Both the NaOH and Na_2CO_3 increase the alkalinity of the solution, which may be quantitively estimated with decinormal acid.

CHAPTER XI.

THE FIXATION OR ASSIMILATION OF NITROGEN.

The "Fixation" or Assimilation of the Free Nitrogen of the Air.—In the processes of decay, putrefaction, and denitrification some of the world's combined nitrogen is destroyed, the nitrogen being set free in the gaseous elementary form. Without the existence of an opposite synthetic process, by means of which the free nitrogen of the atmosphere is brought into combination again, it is obvious that the stock of nitrogen compounds would decrease. To some extent such synthesis or chemical union of nitrogen and oxygen takes place in the air through the agency of lightning and other electric discharges, the nitrous and nitric acids so formed being eventually brought down in the rain. The amount, however, of the combined nitrogen added to the soil annually in this country by showers and dew is very small, being only 4 or 5 lbs. per acre, and a large proportion of it is not derived from free nitrogen, but consists of compounds of ammonia, which have escaped or evaporated into the air from the soil and decaying organic matter.

Berthelot, in some experiments carried out about 1885, showed that bare uncropped soil exposed to the air increased very considerably in combined nitrogen content, even when careful notice was taken of the addition of

nitrogen compounds from the atmosphere. After seven months 50 kilograms of soil showed an increase of 12.38 grams of nitrogen, the amount due to the ammonia and nitric acid in the rain during this time being less than half a gram. Indirect evidence of similar and larger increments has been obtained also from analysis of the soil and of the produce from it during several years, when the crops were grown without nitrogenous manures.

In the first 6 inches of soil of an arable field from 20 to 30 lbs. or more of combined nitogen per acre is added annually, the only source of which is the free nitrogen of the air. From the fact that no gain of nitrogen was found in soils which were first heated to 120° C. in a current of superheated steam, Berthelot concluded that the addition was in some way connected with the existence of minute living organisms in the unheated soil, although he was not able to isolate them satisfactorily. However, through the pioneer work of Hellriegel, Wilfarth, Winogradsky, and Beijerinck, several organisms capable of "fixing" or assimilating the free nitrogen are now well known: the chief of them are species of *Clostridium*, *Azotobacter*, and *Pseudomonas*. There seems little doubt that there are other forms of bacteria besides these which are able to build up nitrogen compounds from the free nitrogen of the air, and evidence exists which appears to indicate that some of the lower fungi are similarly endowed.

The power to utilize gaseous nitrogen is not possessed equally by all the organisms named. *Pseudomonas radicicola* in association with leguminous plants is able to add indirectly large amounts of complex nitrogenous compounds to the land, and these, as well as those manufactured by the other bacteria mentioned, ulti-

mately become of service in the nutrition of ordinary green plants through the processes of decay and nitrifaction. The species of *Azotobacter* add considerable quantities of combined nitrogen also, and a small increase is due to organisms belonging to the genus *Clostridium*.

It is not too much to say that the practical application of the forces possessed by these and similar organisms is a matter of the utmost importance for the production of crops of all kinds, and a complete determination of the conditions under which they carry on their functions to the greatest advantage, is certain to lead to far-reaching effects upon the world's agriculture.

1. **Clostridium pastorianum**, Win.—In 1895 Winogradsky isolated from the soil of St Petersburg an organism which was capable of assimilating free nitrogen when grown under anaerobic conditions in a medium containing dextrose and certain inorganic compounds. A solution containing 40 grams of dextrose was inoculated with a pure culture of the bacillus, and a stream of pure nitrogen was kept passing through it. After twenty days the sugar had disappeared, and analysis showed an increase of 53.6 milligrams of nitrogen in a combined form in the solution. The dextrose was decomposed, 42 to 45 per cent. of it appearing as butyric and acetic acids, the rest being split up with the formation of carbon dioxide and hydrogen. Usually small traces of isobutyl or propyl alcohol and lactic acid are found also.

Winogradsky named the organism *Clostridium pastorianum*. It is a strict anaerobic bacillus, and can assimilate nitrogen in media free from nitrogenous compounds when oxygen is eliminated. It can also fix free nitrogen in solutions even when air is admitted if aerobic species are present to remove the free oxygen and create

suitable anaerobic conditions for its development and growth. A mixture of the *Clostridium* and some aerobic bacteria from the soil when grown in a medium containing 20 grams of dextrose and .01 gram of ammonium sulphate, gave an increase of 24.4 mg. of combined nitrogen, after the dextrose had all disappeared; during the experiment there was free access of air, from which carbon dioxide and nitrogenous compounds had been removed by passing through caustic soda and sulphuric acid.

Clostridium pastorianum belongs to the group of bacteria which produce butyric acid, and resembles its associates in morphology and in many of its physiological powers. It can utilize as a source of energy for its nitrogen-fixing process not only dextrose but levulose, cane-sugar, galactose, and some other sugars. How it obtains the necessary carbohydrate in the soil has not been determined. Two sources appear to be possible, namely, the residues from decaying plant tissues, or the carbohydrate manufactured by minute green algæ so frequently met with in soil. The latter hypothesis is the more likely, and is supported by evidence mentioned later. According to Winogradsky, it does not form butyl alcohol nor ferment mannite, starch, lactose, or calcium lactate, in these respects differing from the common representatives of the "butyric" group. In an active state of growth the organism is a short cylindrical bacillus 1.5 to 2 μ long and 1.2 to 1.3 μ broad, with little or no motile power, and stains yellow with iodine solution. After further development it becomes an oval or spindle-shaped organism of the usual "Clostridium" form, with granular contents; in this stage it contains granulose, a carbohydrate which closely resembles starch,

and like the latter substance stains a violet colour with a solution of iodine (Ex. 40).

Spores arise at the end of the mother-cells, but when fully formed they occupy the middle of the swollen cell, which becomes transformed into a kind of spore capsule. The ripe spores are about 1.6 μ long and 1.3 μ broad, and readily germinate when placed in a sugar medium under anaerobic conditions. Grown on potato or carrot in a vacuum or in nitrogen, the bacillus produces raised greyish-yellow colonies about 1 mm. across, and gives rise to a cheesy odour. The individual bacilli are longer and thicker than they are when cultivated in liquid media, and sometimes develop vibrio or coccus-like involution-forms incapable of spore-formation or nitrogen fixation: long sporeless threads are formed in solutions containing peptone, asparagin, or other nitrogenous compounds, but these die out rapidly. It does not thrive well on sugar agar medium, and refuses to grow on gelatine.

C. pastorianum is widely distributed, but is not present in all soils; it is generally met with in association with aerobic species.

Winogradsky found in soils of South Russia and Paris other forms of species of *Clostridium* with nitrogen-fixing powers. One of these was thicker than *C. pastorianum* (1.6 to 1.8 thick), with spores 1.9 long and 1.5 thick: they all proved difficult to isolate, and were not further studied.

Freudenreich also obtained from Swiss soils an organism which closely resembled Winogradsky's *Clostridium* in all its characters, and had the power of fermenting mannite, which the latter is said not to possess.

Another nitrogen-fixing species is *Clostridium ameri-*

canum isolated by H. Pringsheim. It is a motile organism of the "butyric" group which can carry on its work under less stringent anaerobic conditions than those necessary for *C. pastorianum.* In pure cultures in open flasks it sets up carbohydrate fermentation with nitrogen fixation, and in an atmosphere of hydrogen it is able to ferment starch, mannite, and lactose.

Benecke and Keutner described a large *Clostridium* (*C. giganteum*) from the soil, but its nitrogen fixing power was not tested. The cells are pointed at the ends, and some of them contain two spores. The spores of this species are very large, being 2.5 μ long and 1.5 μ broad.

The power of nitrogen fixation appears to be a common function of many organisms belonging to the class which cause butyric-acid fermentations, but the amount of combined nitrogen added to the soil by the activity of these bacteria is probably small.

For every gram of carbohydrate fermented from 1.2 to 3.7 milligrams of free nitrogen are assimilated.

Ex. 89.—Prepare the following solution:—

Di-potassium phosphate	1 gr.
Magnesium sulphate	.5 gr.
Sodium chloride	A very small crystal of each.
Ferrous sulphate	A very small crystal of each.
Manganese sulphate	A very small crystal of each.
Dextrose	30 gr.
Precipitated chalk	30 gr.
Water	1 litre.

Place 250 c.c. in four separate conical flasks, and inoculate each with .5 gr. of soil from four different localities. Close the flasks with cotton wool plugs, and place them in the incubator

at 22° C. or 30° C. In a few days notice the active production of gas bubbles.

Suck up some of the chalk from the bottom of the flasks with a pipette, spread out on a slide and examine with the microscope :

(1) Are Clostridium forms present ?

(2) Run under cover slip some iodine solution. Do the bacteria stain with a blue colour ?

(3) If spores are present stain as in Ex. 12.

(4) Try and obtain growths of any of the organisms present on potato under anaerobic conditions. (Buchner's tube method.)

2. **Species of Azotobacter.**—In 1901 Beijerinck isolated from soil and canal water two species of organisms which are able to assimilate the free nitrogen of the air and build up proteins and other nitrogen compounds needed for the growth and development of their bodies. These he included in a genus which he named *Azotobacter*. By their multiplication they are able to add considerable amounts of combined nitrogen to the soil, which ultimately becomes available to farm and garden crops through the processes of decay and nitrification. Since Beijerinck's discovery in 1901 much attention has been devoted to the determination of the distribution, growth, and conditions essential for nitrogen fixation by these organisms. The different species are readily isolated from almost every kind of soil except those which are acid or of dry, sandy character. They are also commonly present in well and river water and in sea water, and are found on the surface slime of species of *Fucus*, *Laminaria*, and other algæ. Freudenreich and Thiele found them in the soil down to a depth of 50 or 60 cm.; at 100 to 190 cm. deep *Clostridium pastorianum* was met with, but no species of *Azotobacter*.

As in the case of *Clostridium*, these organisms obtain the energy they need to bring the inert free nitrogen into chemical combination from the combustion of carbon compounds in the soil. The presence of phosphates and lime is necessary for their growth, and possibly potash and other inorganic salts are useful also; according to Fischer's investigations they die out or become inert in soils containing less than .1 per cent. of lime. All the species of *Azotobacter* are strongly aerobic and need an abundant supply of air. An adequate amount of moisture is also essential for their welfare, the best results being obtained when the soil contains between 5 to 15 per cent.; more than this leads to imperfect aeration. They only thrive satisfactorily when nitrogen compounds are present in small amount or absent altogether. They carry out their work most effectively at a temperature between 25° C. and 30° C., but growth is active at 20° C., and A. Koch states that he found nitrogen fixation going on in the soil during winter. At temperatures above 30° or 35° C. they produce involution forms and become weakened in vitality and nitrogen-fixing power. To avoid complications with *Clostridium pastorianum* present in the soil upon which he experimented, Beijerinck used mannite and calcium, sodium or potassium salts of propionic acid as the necessary carbon food for these organisms, since these substances do not readily undergo the "butyric" fermentation. He found Azotobacter also capable of utilizing glucose, levulose, galactose, cane sugar, and salts of several organic acids as sources of carbon.

The nutrient medium in which the cultures are made is placed in small amounts in wide-bottomed flasks, so as to allow a free access of air to a large surface of the

solution. With 20 grams of glucose or mannite in a litre of nutrient solution Freudenreich found that *Azotobacter chroococcum* assimilated or "fixed" from 12 to 24 mg. of nitrogen in two or three weeks. When grown on gypsum plates with less liquid present and abundant air supply 160 mg. of nitrogen were assimiliated in the same time. By cultivating it upon agar mannite-phosphate plates A. Koch obtained an increase of 180 mg. of combined nitrogen per litre of medium.

Gerlach and Vogel found that the amount of nitrogen assimilated is influenced largely by the amount of glucose present in the nutrient solution. From 1 to 12 grams of sugar per litre proved beneficial, and the amount of nitrogen assimilated increased proportionally; 15 grams checked the action of the organisms to some extent. The following are some of their results after five weeks' growth:—

	Milligrams of Nitrogen fixed.
With 1 gr. of grape sugar present . .	7.4
" 3 " " " . .	17.3
" 5 " " " . .	39.4
" 10 " " " . .	91.4
" 12 " " " . .	127.9
" 15 " " " . .	62.9

They also showed that the power of nitrogen fixation decreases with the age of the culture, as indicated below, and is reduced by cultivating the organisms for several generations in nutrient media:—

	Milligrams of N. in 1 litre.
A culture 18 days old "fixed" . .	127.9 in 5 weeks'
" 110 " " . .	54.9 "
" 328 " " . .	23.4 "

All the organisms belonging to the genus *Azotobacter* are extremely variable in size and shape. The typical form is a short oval cell from 4 to 6 μ long and 3 to 4 μ broad (Fig. 32). Under certain conditions these cells become thicker and rounded into a coccus, two of which are usually found united—a diplococcus. They appear also as streptococci, and can divide in two or three directions so as to form sarcina-like "packets"; the latter are especially abundant in old cultures. Spores are not known. The cell walls are often thick and slimy and stain well with methylene blue or gentian violet. The protoplasm within contains small refractive granules and usually a vacuole. Iodine stains the organisms a yellowish tint. Four or five species or varieties have been described. Although they differ in nitrogen fixing power, they are probably modifications of one organism. The following are the best known :—

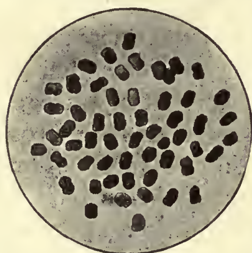


FIG. 32.—*Azotobacter chroococcum*, Beijk. (×500).

A. chroococcum, Beijk.—This, the first species isolated, is very widely distributed in field and garden soils. It produces after a time a brown or blackish coloration on solid substrata and on the surface of liquid media. In young cultures the cells are motile, short, plump rods, 4.5 to 7 μ long, and 3 to 4 μ broad, generally in pairs. On agar-mannite plates (Ex. 91) at 30° C. the colonies are pasty at first, round, white, usually with dark concentric rings in the middle: later they become dark brown, and may grow to be 5 or 6 mm. in diameter The cells in three or four days are short rods with rounded ends, often in pairs, 4 to 5 μ in diameter; others are longer oval rods, 3 to 6 μ long and 2 to 3 μ thick. In a week or ten days they may

become smaller and arranged in short chains, three to eight or more together; the "sarcina" form appears in abundance when the colonies become brown. In liquid media the pigmentation appears usually in four or five weeks from the time of inoculation where the medium is kept at 20° to 25° C.

A. agilis, Beijk., originally isolated from canal water, is a large actively motile organism with a bundle of polar cilia. The cells are oval, transparent, with distinct cell-walls, granular protoplasm, and vacuoles. It grows well on an agar medium containing glucose in place of mannite (Ex. 91). On such medium with ½ per cent. of calcium propionate in place of the sugar, the colonies of *A. agilis* becomes surrounded by a greenish yellow fluorescent zone which is characteristic of the species.

A. vinelandii, Lipman, a form isolated from some North American soils, produces a thick white membrane on mannite solutions, which becomes yellowish. Ultimately the liquid becomes yellowish-red in colour. On mannite agar the colonies are semi-transparent at first, rounded, and 2 to 4 mm. in diameter: they produce a yellowish pigment, which diffuses through the medium.

A. Beijerinckii is another species or form isolated by Lipman from American soils. The organisms are spherical, non-motile, and much larger than the preceding kinds; in mannite solutions they occur singly or in short chains.

When grown in mannite solutions a white deposit is produced, and small white colonies appear on the surface of the liquid and on the walls of the flask.

The colonies on mannite agar are well developed in four or five days, somewhat rounded, white, and moist, with netted surfaces.

Ex. 90.—Grow and isolate species of *Azotobacter* from soil. Prepare the following solution:—

Di-potassium phosphate	.2 gr.
Mannite	20 gr.
Tap water	1 litre

Add 100 c.c. of the solution to a conical wide-bottomed flask, and inoculate with .1 to .2 gr. of garden soil. Incubate at 28° to 30° C. in the dark. In two or three days a scum or pellicle forms which consists of a mixture or zoogloea of small bacteria and *Azotobacter* with amœbae and infusoria. Examine a small portion of the scum for stumpy or oval rods of *Azotobacter*. Transfer a drop or a small portion of the scum from this flask to another flask of the sterilized nutrient solution and incubate: later transfer a drop from the second to the third flask. In this way a tolerably pure culture of *Azotobacter* may be obtained in three or four generations. From it quite pure cultures may be prepared by isolation on mannite agar as indicated in the next Exercise.

Ex. 91.—Prepare the following mannite agar medium:—

Di-potassium phosphate	.02 gr.
Mannite	2 gr.
Agar	2 gr.
Distilled water	100 c.c.

Sterilize. Add 10 to 20 c.c. to a Petri dish and slowly cool, turning the dish upside down, so as to allow the water of condensation to collect on the lid and not on the surface of the medium. Add a small portion of the membrane or a drop of the solution from the flasks in the previous Exercise to a small quantity of sterile distilled water, and pour some of it over the surface of the plate. Incubate at 28° C., and note the colonies which grow: examine the size and shape of the organisms from each kind of colony.

Test the action of the organisms from the different colonies in "bouillon" at 20° C. This medium remains sterile or clear when inoculated with pure *Azotobacter*, but generally becomes turbid when other organisms are added.

Ex. 92.—*Azotobacter* colonies grow well on gypsum plates which are permeated by a mannite phosphate solution.

Take some plaster of Paris (gypsum) and mix it with water to

the consistency of thick cream (about eight volumes of gypsum powder to three of water are needed); pour it on a plate of glass so as to be about a quarter of an inch thick, and before it has set cut it into narrow strips 3 inches long by ½ in. to ¾ in. broad, or into circular pieces which will fit easily inside an ordinary Petri dish.

Prepare the following solution:—

Di-potassium phosphate	.05 gr.
Sodium chloride	.05 gr.
Mannite	2 gr.
Water	100 cc.

Add about 5 c.c. to some test-tubes, and place in each of these a strip of gypsum, so that the lower end dips into the solution: or pour a few c.c. into a Petri dish, and put in a circular plate of the plaster: do not wet the upper surface of the latter. Then place a drop or two of a culture of *Azotobacter* on surface of the gypsum plate, and incubate at about 28° to 30° C. Observe the greyish-white growth, which becomes dark brown generally in three or four days.

Ex. 93.—Isolate *Azotobacter* from soil by the following method (Gerlach and Vogel):—

Take 20 grams of fresh garden soil and place in a large Petri dish: pour on it 100 c.c. of the following solution:—

Grape sugar	1 gr.
Di-potassium phosphate	.25 gr.
Sodium chloride	.25 gr.
Calcium carbonate	.25 gr.
Ferrous sulphate	a trace
Water	500 c.c.

Keep it in the dark at 28° C. for two or three days. Examine the scum which arises for cells of *Azotobacter*: these are usually abundant and crowded into a slimy zoogloea.

(i) With a platinum needle transfer a small portion of the scum to a drop of water on a glass slide, cover with a slip, and press the latter so as to break up the zoogloea.

(ii) Run under the cover slip a drop of iodine solution, or mount a small piece of the scum in the latter. Observe the yellow colour of the *Azotobacter* cells, and look out for violet-stained *Clostridium* cells, which are generally present.

(iii) Inoculate a plate of the following agar medium :—

Agar	2 gr.
Grape sugar	.2 gr.
Di-potassium phosphate	.2 gr.
Water	100 c.c.

The colonies of *Azotobacter* are at first colourless and shining; later they become elevated and yellow, and finally, in five or six days, turn a deep brown colour.

If no *Azotobacter* organisms are seen on first examination of the scum as above, add 1 c.c. of the liquid from the mixed soil and culture solution to 50 c.c. of the nutrient solution, and incubate for two or three days at 28° C. Another grape-sugar plate can then be inoculated with a drop of this culture, and the *Azotobacter* colonies afterwards obtained.

3. It is hardly likely that the only organisms capable of assimilating the free nitrogen of the air are those already mentioned belonging to the genera *Clostridium* and *Azotobacter*, although Winogradsky and others obtained negative results with many bacteria which they specially investigated from this point of view. There is indeed some evidence that a number of species of soil organisms are able to effect the synthesis of nitrogen compounds from the free nitrogen of the air in slight degree, but the difficulties connected with the investigation of the problem are great. In most cases the increase in nitrogen content due to nitrogen fixation is necessarily very small, and frequently lies within the limits of experimental error, so that much caution must be exercised in accepting or rejecting results.

Alinit.—About 1895 Caron isolated from the soil a bacterium, *Bacillus ellenbachensis a*, and which in pure cultures was offered for sale under the name "Alinit." It was claimed that this organism when applied to poor soils was capable of "fixing" the nitrogen of the air, which ultimately became of service for the nutrition of cereal crops. Experiments to test the beneficial influence of "Alinit" have led to conflicting results, Stoklasa, Grandeau, Malpeaux, and others maintaining that infected plots frequently produced much larger crops than plots which were untreated, while many experiments on barley and other crops carried out in different parts of Europe showed no increase in yield after the application of "Alinit" cultures. *Bs. ellenbachensis* belongs to a common group of soil bacteria characterized by their large size, aerobic nature, and power of spore production; the group includes the hay bacillus (*Bs. subtilis*), *Bs. mycoides*, *Bs. megatherium*, and some others closely resembling these. It is a putrefactive species, which decomposes proteins with the formation of peptones, amino-acids, and other compounds, and under certain conditions brings about the reduction of nitrates to nitrites. According to Breal and Bokorny, leucine, tyrosine, and other similar nitrogenous substances produced in putrefactive processes can be utilized for nutritive purposes by green plants of various kinds, so that the observed beneficial effects of the "alinit" bacterium may be due in some cases to its power of breaking down the insoluble nitrogen compounds and rendering them available to crops. In addition to the power just described, Stoklasa maintains that the "alinit" bacillus (which he considers is identical with *Bs. megatherium*, De Bary) is able to assimilate free nitrogen when living

in media or soil containing little or no combined nitrogen, provided that xylose, galactose, or other carbon compounds are supplied.

The "Alinit" Bacillus (*Bs. ellenbachensis, a*, Caron) is a large rod-shaped organism, which does not form very long threads as a rule. Each cell has rounded ends, and is from 3 to 6 μ long and 1.25 to 1.5 μ thick; it is stained by Gram's method. The organism is motile, and forms central oval spores, which exhibit distinct polar germination, the young rod escaping from the end of the spore. The optimum temperature for growth is about 28° to 30° C., and it is aerobic.

Gelatine.—The colonies are at first roundish, somewhat dark in the centre, with radial filamentous outgrowths; later the gelatine is liquefied. The "stab" soon shows funnel-shaped liquefaction, with a pellicle on the surface and a flocculent deposit.

Agar.—Surface colonies yellowish-white, roundish or slightly granular and puckered, surrounded by hair-like extensions; deep colonies with irregular thread-like projections, which resemble the hyphæ of fungi.

Potato.—Greyish-white growth, soft and flat.

Milk.—Milk is coagulated, and becomes alkaline, with a surface scum; the precipitated casein is peptonized and slowly dissolved.

3. An aerobic spore-forming bacterium capable of assimilating free nitrogen was isolated from the soil around the nodules of *Vicia villosa* and other leguminous plants by Löhnis and Westerman; they named it *Bact. danicus.* It is very variable in form and size, appearing in mannite solutions as a large coccus or as a rod 3 to 5 μ long and 1 μ thick. On mannite-agar it produces spores sometimes in "clostridial" cells, the colonies on this medium being round, shining greyish-white 2 to 4 mm. in diameter in five days at 20° C.

The growth on potato very closely resembles that produced by *Bs. vulgatus*.

The organism stains irregularly with fuchsin.

4. Frank, Hellriegel, and others observed that unsterilized soils which become covered with minute green algæ frequently increase very considerably in nitrogen content, but when protected from the light, or when the surface is covered with sand or other material to prevent the growth of these small plants, such soils do not become richer in combined nitrogen. Schloesing and Laurent found that free nitrogen of the air was absorbed and fixed when the latter was enclosed in a vessel containing soil covered with a coat of green algæ and exposed to light. The accumulation of nitrogen compounds produced under these conditions may amount to .8 gr. for 1 kg. of soil, and occurs chiefly in the thin layer near the surface. Such observations led to the belief that minute green algæ belonging to Cyanophyceæ and Chlorophyceæ are able to utilize free atmospheric nitrogen, but Kruger and Schneidewind and Kossowitch, by careful experiments with pure cultures of *Cystococcus*, *Stichococcus* and *Chlorella* in media from which combined nitrogen is eliminated have proved that this view is incorrect. No growth occurs when free nitrogen is supplied to such cultures, but there is luxuriant development when nitrates are provided. Bouilhac obtained similar results with cultures of *Nostoc punctiforme*. It has been found, however, that when soil bacteria are added to pure cultures of these algæ nitrogen fixation goes on extensively. Between the algæ and the bacteria there is no doubt a kind of symbiosis; the latter organisms fix the nitrogen, deriving the organic compounds essential for the process from products of carbon assimilation carried on in the light by the green algæ,

the algæ in turn obtaining some or all the nitrogen they need for nutrition from their associates.

Stoklasa states that the "Alinit" bacillus fixes atmospheric nitrogen in considerably larger amounts in the presence of species of *Stichococcus* and *Nostoc* than when these algæ are absent. He also notes that soils containing an abundance of algæ are richer in bacteria than those which are poor in algæ, and that *Pleurococcus* and *Nostoc* contain 6 to 7 per cent. of pentose, 70 per cent. of which is soluble in water, and easily available for the nutritive requirements of bacteria.

CHAPTER XII.

THE FIXATION OR ASSIMILATION OF NITROGEN—(*Continued*).

1. **The Fixation of Nitrogen by Bacteria in Symbiosis with Leguminous Plants.**—It has been known among agriculturists from the earliest times that the growth of a leguminous crop, such as beans or clover, leaves the land in a high condition for the growth of a subsequent crop of wheat or other cereal. It was recognized that in some way or another a leguminous crop differed very much from any other class in its effects upon the land, and its cultivation was to a large extent found to be equivalent to the application of a considerable amount of dung or other manure. Experiments carried out in the open field at Rothamsted have shown that the growth of a leguminous plant leaves the ground richer in nitrogen than it was before, and this in spite of the fact that the crop itself is highly nitrogenous in composition. Below are given the average annual amounts of nitrogen per acre obtained in the several crops mentioned, grown on the land every season during a period of about twenty-four years: the soil was manured with phosphate, potash, and other mineral manures, but no nitrogen was applied:—

	Average amount of Nitrogen per Acre per Annum.
Wheat	22.1 lbs.
Barley	22.4 lbs.
Beans	45.5 lbs.
Clover (22 years only)	39.8 lbs.

From this table it is seen that the annual amount of nitrogen in a crop of beans or clover is more than double that in a cereal crop grown under similar conditions side by side on the same field. In Little Hoos Field, Rothamsted, barley was grown in 1873 adjoining a plot of clover: both received the same treatment, and the soil was uniform in character. The amounts of nitrogen obtained in the two crops were :—

	Lbs. of Nitrogen per Acre.
In the barley	37.3
In the clover	151.3

In the following season barley was sown on both plots, the nitrogen obtained in the crop being :—

	Lbs. of Nitrogen per Acre.
In the barley after barley .	39.1
In the barley after clover .	69.4
Difference .	30.3

From the figures given above it is seen that the clover contained more than four times as much nitrogen as the barley growing side by side with it in the same field: moreover, the soil of the plot from which the clover was cut was left so much richer in nitrogen than that on which barley had been grown that another barley crop in the following year was able to obtain over 30 lbs. more of nitrogen from it than from the latter plot. Chemical analysis also showed an increased nitrogen-content in the soil which had grown a clover crop over that from which barley had been harvested.

Herr Schultz obtained results of a similar nature on a

large scale on his estate at Lupitz in Germany. The land there is of a light sandy character, and about half a century ago was too poor to grow a profitable crop of oats or rye without an expensive application of nitrogenous and other fertilizers. However, after the growth of lupins or serradella, manured with superphosphates and kainit, the barren soil has improved so much that large yields of cereals as well as leguminous crops have been obtained annually from it for over fifty years in succession, and the land has become three times as rich in nitrogen as it was at first, although no nitrogenous manure has been applied during this long period.

Results such as these created a special interest in the investigation of the sources from which leguminous plants obtain their stock of nitrogen, and many distinguished workers applied themselves to the subject during the last half of the nineteenth century.

2. When wheat, mustard, or other non-leguminous plants are grown in plots containing field or garden soil of known chemical composition, the total amount of nitrogen in the fully developed plants and the soil at the end of the experiment is equal to that contained originally in the soil and the seeds which were sown. In the case of beans, however, and vetches or other leguminous plants grown in the same soil under similar conditions, it is found that the nitrogen present in the soil and the plants when fully grown is very much greater than that which was supplied at the beginning of the experiment in the soil and seed. They are able to accumulate large quantities of nitrogen in a manner not possessed by cereals or other crops, and from a source which is evidently not available to the latter. Acquaintance with facts of this kind suggested the

atmosphere as the source from which leguminous plants draw their store of nitrogen. Since the amount present in the form of ammonia and oxides of nitrogen was found to be very small and quite insufficient to account for the quantity collected by leguminous crops, chemists and physiologists were driven to consider the free nitrogen of the air as a possible nutrient substance, although on *a priori* grounds it appeared unlikely that plants could assimilate and bring into chemical combination an element of such inert and indifferent nature.

The careful investigations of Leibig and Boussingault in the first half of last century appeared to support the view that plants could not utilize free nitrogen; but between 1850 and 1857 Georges Ville in France carried out some experiments with leguminous plants grown in natural soil in closed glass cases, to which only air which had been carefully deprived of any combined nitrogen had access, and found that they grew luxuriantly under these conditions and contained more nitrogen than was originally present in the soil and seed used. He concluded that the plants were able to assimilate free nitrogen, and although it was suggested that small amounts of ammonia had been inadvertently supplied to the plants in the distilled water used for watering them, Ville convinced himself by repeated experiments that his conclusion was correct.

Later, Lawes and Gilbert in England carried out experiments upon peas, vetches, and lupins, growing them in carefully sterilized sand, to which were added phosphates, potash, and other necessary mineral food-constituents, but no combined nitrogen: air, however, with its free nitrogen was admitted to the cultures. In all cases their results were negative; the plants under

these conditions soon suffered from nitrogen hunger and remained small and stunted in growth. It was not until about 1886 that the question of the assimilation of free nitrogen by leguminous plants was solved by the investigations of Hellriegel and Wilfarth in Germany. Any doubt about the problem was removed, and a satisfactory explanation was furnished of the diametrically opposite results obtained by Ville and Lawes and Gilbert. It was discovered that peas grown in sterilized soil are dependent on combined nitrogen and die of nitrogen hunger when the amount supplied is small, just as cereals do; neither peas nor wheat are able to make use of the free nitrogen of the air under these conditions. They learnt, however, that after an addition to the sterilized soil of a few cubic centimetres of a watery extract of soil from a field which had grown peas, the failing wheat plants were in no way benefited, but the drooping pea plants recovered rapidly and increased greatly in nitrogen-content, the free nitrogen of the atmosphere supplying all their needs in respect of this element. Upon the roots of the peas to which the extract of the soil had been applied were seen a number of warty excrescences termed *nodules* (Fig. 33), but on those grown entirely in sterilized soil none were found. A direct relationship was found to exist between the possession of root nodules and the assimilation of free nitrogen by leguminous plants. With them the plants thrive luxuriantly, and nitrogenous compounds accumulate in all their tissues, even when no other source is available for nitrogen than that present in a free state in the atmosphere: without them growth is limited unless combined nitrogen is supplied. The fact that sterilization of the soil or heating the watery soil extract to

70° C. or over entirely checked the formation of nodules suggested that living organisms were in some way responsible for the appearance of these peculiar structures, and since the researches of Hellriegel and Wilfarth the soil bacteria which stimulate the production of root nodules have been isolated and extensively studied.

FIG. 33.—Root of runner bean (*Phaseolus*) showing nodules.

The evidence obtained by Hellriegel and Wilfarth of the assimilation of free nitrogen was indirect: they showed a large gain of nitrogen in plants grown under conditions in which no other source than that of the free nitrogen of the air was available. In 1890, however, direct proof of the removal of free nitrogen from the air and its fixation in the plant was obtained by Schloesing and Laurent. They grew leguminous plants in closed vessels and made analyses of the air at the beginning and end of the experiment: the amount of nitrogen taken from the air was found to correspond very closely with the increase of nitrogen in the crop.

3. The form, number and arrangement of the root-nodules upon the different species of leguminous plants varies considerably. Those of lupins are large, com-

paratively few in number and attached by wide bases to the tap root and stronger lateral roots. In the case of many species the nodules are found in large numbers on the smaller roots to which they are attached by narrow necks. Some of them, such as those upon *Robinia*, *Trifolium*, *Onobrychis*, and *Medicago*, are elongated and not infrequently branched: while those of

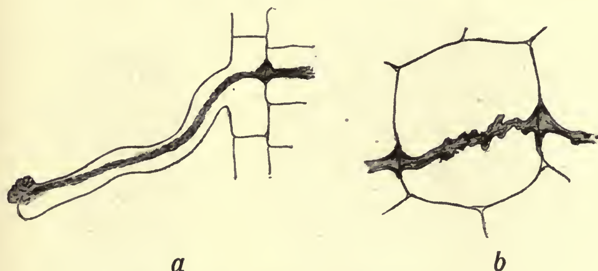


FIG. 34.—*a* Root hair of pea showing *infection-thread*.
b Infection-thread passing through cell of young nodule.

Anthyllis, *Serradella*, and *Phaseolus* are usually small round structures.

The nodules are caused by the soil organism *Pseudomonas radicicola* (Beijk.), Moore (*Bacillus radicicola*, Beijk.), which penetrates into the young roots chiefly through the root hairs: in lupins and some other species infection apparently takes place through the older parts of the roots as well. After entry the organism multiplies and forms a filamentous zoogloea, — the *infection-thread*—which resembles a fungus hypha so closely that it was at first confused with the latter. The infection-thread consists of small rod-shaped bacteria imbedded in a gelatinous substance from which they are difficult to differentiate. It can be traced along the interior of the root-hairs, and through the cells of the cortex. Where

it passes through the cell-walls it generally exhibits peculiar trumpet-shaped expansions (Fig. 34). Here and there upon it are minute swellings from which bacteria appear to escape into the cell-cavity which they subsequently fill by repeated division and growth.

The nodules arise endogenously from division of the infected cortical cells near the endodermis of the rootlets.

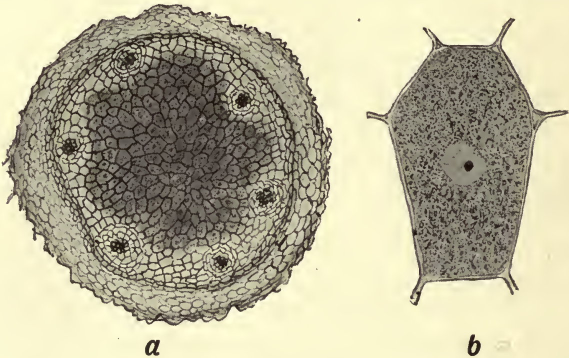


FIG. 35.—*a* Transverse section of nodule from red clover root showing dark bacteroidal tissue in centre, with cortical tissue and vascular bundles round it (×20).
b Single cell of bacteroidal tissue (×250).

Occupying the central part of a fully developed specimen is a mass of parenchyma filled with bacteria or *bacteroids* (Fig. 35). Surrounding the *bacteroidal tissue*, which is often pinkish or greenish in colour, are small vascular bundles connected with those of the root, upon which the nodule is growing: outside is the cortex and epidermis. The bacteroidal tissue is a meristem, which by rapid division in all directions alike results in the production of a more or less spherical nodule: frequently, however, division goes on chiefly at the end of the tissue farthest away from the root, in which case the nodule

becomes elongated. The infection-threads soon extend into the new tissue, and although they do not always come into direct contact with the nuclei of the cells through which they pass, the latter appear to be influenced by the presence of the bacteria in the thread, since they increase in size and develop an abnormal number of nucleoli, after which they finally degenerate. In clover and some other plants the infection-threads remain visible for some time before becoming obliterated.

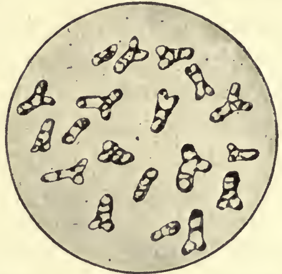


Fig. 36.—Bacteroids from red clover nodule (× 1000).

The organisms present in the infection-threads and youngest parts of the nodules are short straight rods. In the older parts they become distributed through out the interior of the cells, grow considerably in size and thickness, and undergo change into branched, curved or T and Y shaped forms, commonly termed *bacteroids*, in which state they stain irregularly, and appear to consist of vacuolated protoplasm (Fig. 36). The bacteroids soon lose their power of division, and after a time are dissolved by a proteolytic enzyme secreted from the surrounding protoplasm, the compounds produced being transferred to the flowers and ripening seeds of the leguminous plant. The nodules eventually shrivel and decay, but some of the small rods remain unchanged and find their way into the soil ready to infect a subsequent crop.

The relationship between the bacteria and the leguminous plant is one of symbiosis or mutual assistance. At first the bacteria live as parasites, deriving all their

food from the leguminous plant as well as the carbohydrates essential for the supply of energy needed in the nitrogen fixing process. Starch is generally present in the tissues of the nodules, especially in autumn and during the resting period of the plant. It is possible that the organisms in some cases live altogether as parasites without offering anything in return for the nutrient substances which they receive from their host, under which circumstances the plant may exhibit little or no increase in nitrogen content. Usually, however, the nodules grow in size, vast numbers of bacteroids are produced, and large amounts of nitrogen are obtained by them from the air, and used for the formation of nitrogenous compounds, of which their bodies are mainly composed. After a time the bacteroids become disorganized and dissolved, the compounds in solution being absorbed and utilized by the leguminous plant for its own nutrition, and especially for the formation of its seeds, the plant thus becoming parasitic upon the bacteria.

The facts of the assimilation of the free nitrogen of the air are clear, but the manner in which the process is carried out by the combination of plant and bacteria is still obscure. There is apparently no increase of nitrogen in the soil itself on which leguminous plants are growing, the excess obtained on analysis of the soil after the growth of a crop of beans or clover being due to the root residues left in the ground after the plants are cut and harvested. Nobbe and Hiltner and others have shown that the gaseous nitrogen is absorbed by the nodules themselves, and not by the other parts of the plant: plants grown in water culture with the root nodules immersed gain no nitrogen, and not until the latter are brought above the

surface of the liquid into the air are they of service to their symbiotic partner.

The greatest amount of nitrogen assimilation takes place at the time when the formation of bacteroids has reached a maximum, at which stage the plants are just beginning to flower; during the ripening of the seed the organic nitrogenous compounds are removed from the nodules, and their nitrogen content becomes reduced to near that of the rest of the plant. Stoklasa found that lupin nodules in which the bacteroidal tissue was fully developed contained 5.2 per cent. of nitrogen, most of which was present in the form of proteins: the plants were then in flower. Later, when fruit and seeds were ripe, the nodules contained only 1.7 per cent., an amount about equal to that normally present in the unaltered parts of the roots. Analyses of the nodules of peas and dwarf beans by Frank showed a nitrogen content of about 7 to 7½ per cent.

Mazé and Golding have shown that the nodule bacteria are able to assimilate the free nitrogen of the air to a limited extent when grown in pure cultures apart from the leguminous plant, and it is possible that under certain favourable conditions, where a suitable supply of carbohydrates or other organic carbon compounds is available, they may be able to do the same during their existence as separate isolated agents in the soil. But very little is known of their life and nutrition in the soil. Nodule-forming bacteria are ubiquitous, and it is exceptional to find soil in which nodules are not formed on one or another species of leguminous plant, even in cases where the land has carried no crop of this class for forty or fifty years. Certain poor soils, however, are met with in which the specialized organisms adapted to lupins,

serradella, and some other species of leguminous plants are missing, and until they are supplied either in soil from a field which has grown a good crop of these plants or in pure cultures, such plants grow very unsatisfactorily.

4. As already indicated, there is considerable variety in the morphology of the nodule organisms, but all the forms are doubtless modifications of a single species produced by variation in environment. Many of the forms exhibit striking differences also in regard to their power of producing nodules. The most luxuriant growth of these root excrescences upon any particular plant is brought about in the speediest and most effective manner by inoculation with cultures derived from nodules of the same species of plant. Thus upon the roots of peas they are most easily produced by organisms derived from pea nodules, although their formation can be stimulated by those obtained from beans and vetches. Those taken from the roots of *Robinia* are active upon beans and certain clovers. Several varieties, such as those obtained from lupins, clovers, and serradella, are somewhat exclusive in their behaviour, and do not affect plants belonging to other genera. The bacteria from peas produce nodules upon beans, but not upon lupins, nor upon plants belonging to the genera *Trifolium*, *Medicago*, *Anthyllis*, or *Ornithopus*. Although what may be termed the virulence of the organism, or its prompt and unfailing power of nodule production, is largely dependent upon the origin of the bacterium, it is also greatly influenced by nutrition; pure cultures which have been grown for some time upon gelatine or media containing considerable amounts of combined nitrogen lose much of their power in this respect. The com-

position of the soil and its chemical reaction influence nodule formation, and affect the vitality and virulence of the nodule bacteria. The latter are rarely abundant in acid soils, and for the healthy exercise of their physiological powers they need an adequate supply of mineral substances, especially compounds containing lime, phosphates, and potash; without these their virulence is reduced and their ability to assist the leguminous crop is diminished. These facts are of great importance in the preparation and use of pure cultures for agricultural purposes.

5. **Soil inoculation.**—From what has been said previously it is obvious that the growth of a good leguminous crop is of the greatest importance to the farmer. In itself it provides valuable food for farm stock, and at the same time leaves the land well supplied with nitrogen, which vastly assists the growth of cereals and other crops succeeding it. The certain production of a good leguminous crop is therefore very desirable. In most cases the sowing of the proper seed is enough to ensure success, the nodule bacteria being very abundant in the majority of soils. In some soils, however, certain kinds of leguminous plants have been found to grow very poorly: no nodules have appeared upon their roots, and the land has been no more benefited than it would have been by the growth of a cereal crop. In such instances the right nodule-organism is missing from the land, and its introduction is necessary before satisfactory results can be expected. Experiment has shown that this can be achieved by taking soil from a field which has grown a good crop of the particular plant in question, and spreading it over the land on which the crop has failed. Such a process of

soil inoculation very frequently has given good results, but it is a laborious and expensive process. To avoid these disadvantages, Nobbe, in Germany, conceived the idea of inoculating the soil or the seed before sowing with pure cultures of organisms from the several leguminous crops usually cultivated on the farm. The pure cultures were grown upon agar and gelatine media impregnated with decoctions of the leaves of the various legumes, and sold under the name of *Nitragin*. The usual method of using the latter was to transfer the culture to water or milk, and wet the seeds with the liquid. When dry the seeds were sown, the right organisms being necessarily supplied to the soil at the same time. Hundreds of trials were made with "Nitragin" in most European countries, and although in a small percentage of cases the inoculated seed produced larger yields of crop than untreated samples, the majority of the trials proved futile : the soil contained plenty of the nodule organisms for infection of the young leguminous seedlings, and to add more was superfluous.

In 1904, Dr G. T. Moore, in America, came to the conclusion that the failures of Nobbe's "Nitragin" were largely due to the loss of vitality or virulence of the nodule organisms contained in it, a result brought about by cultivating them upon media rich in nitrogen. There is little doubt that the nitrogen-fixing power and power of infecting the roots of legumes is greatly reduced by the presence in the soil of nitrates and other nitrogenous compounds, and it is likely that the virulence of the organisms is also checked by cultivation on artificial media containing considerable amounts of combined nitrogen. Moore grew the organisms in liquid media containing very small amounts of nitro-

genous compounds. Pieces of cotton wool were soaked in the culture and then dried ; in a dry state attached to cotton wool the organisms retain their vitality for a year or two. Along with the infected cotton wool two packets of nutrient salts were supplied, one containing sugar, magnesium sulphate, and potassium phosphate, the other ammonium phosphate. The first packet was dissolved in water, and the cotton wool then added. In this solution the common bacteria of the air and water do not grow freely, but the nodule organisms readily multiply in it. After twenty-four hours the ammonium phosphate is added, to encourage further development of the nodule bacteria. If growth is satisfactory the liquid becomes opalescent or milky, after which it is used to sprinkle over the seeds ; these are then allowed to dry, and are sown afterwards in the ordinary way.

More recently cultures similar to those of Moore have been issued under the name *Nitro-bacterine.*

The practical utilization of pure cultures of the "nodule-forming" organisms is still in the experimental stage, in spite of the large amount of attention which has been given to the problem. It is quite certain that in certain cases seed inoculation in the manner described above has been very beneficial and remunerative, but exact knowledge of the conditions which ensure an increased yield after such inoculation is still wanting. The evidence seems to point to the conclusion that on much of the cultivated land in Europe the use of "nitragin" or "nitro-bacterine" is quite unnecessary for the growth of good crops of the ordinary leguminous plants, the soil being already well supplied with organisms of the right kind for the adequate infection of the roots of the crop. Failure to obtain increased

returns after inoculation seems to occur where the land is supplied with an excess of easily assimilated nitrogenous compounds which check the growth of the nodule organisms, and also on acid soils and those deficient in phosphates and potash.

Inoculation is likely to be effective upon soils poor in nitrogen, especially where leguminous crops have not been grown previously, and also on land which has given meagre crops of this class, with roots devoid of nodules.

Much careful and continued experiment is needed to determine the best kinds of leguminous crop to grow, how to grow them, and how to use them, so as to bring to the farm the greatest amount of nitrogen at the least expense. These are problems which should be considered along with the cultivation and application of pure cultures of the various nodule organisms if the work is to be of economic interest.

Pseudomonas radicicola (Beijk.), Moore.—*Ps. radicicola* is an aerobic bacterium without spores, and grows best at about 15° C., being easily killed at a temperature of 60° to 70° C. As previously indicated, the size and form of the organisms present in the nodules of the Leguminosæ vary with the condition of development of the nodule, and the kind of plant from which they are taken. In the infection threads and young parts of the nodule, the bacteria are always short non-motile rods about 1 to 2 μ long and .5 to .6 μ broad. The bacteroids differ considerably in different plants. Those of *Phaseolus*, *Hedysarum*, and *Genista* are thickish rods, straight or curved, but rarely branched: in *Vicia*, *Pisum* and *Trifolium*, when fully developed, they become T and Y shaped, 2 to 4 μ long, .5 to .8 μ broad, while those of *Lupinus* are often extensively and irregularly branched.

When grown on artificial media the nodule organisms are at first minute rods, single or in pairs, actively motile, each possessing a single polar flagellum, hence their inclusion in the genus *Pseudomonas*. Later they lose their motile power and change into bacteroids, becoming irregular in form, curved, and sometimes more or less branched. They often excrete a mucilaginous substance; the protoplasm is of unequal density and does not stain uniformly.

Wood-Ash-Maltose solution.—In this solution the organisms flourish, the medium becoming turbid and ropy with a whitish growth in it which accumulates on the bottom of the flask.

Agar.—On Wood-ash-maltose agar (Ex. 99) plates the surface colonies are round, raised, with a translucent appearance very like drops of melted paraffin wax. They may grow to a diameter of 2 or 3 mm. in five to ten days, and at first are watery, but after a time develop a slimy substance which can be drawn out into long threads. The deep colonies are smaller, round, oval or triangular, granular and whitish.

Ex. 94.—Dig up well-grown specimens of beans, red clover, alsike, kidney-vetch, birds'-foot trefoil, sanfoin, and other leguminous plants just before they flower: wash the roots carefully, and make observations upon the number, position, size, form and colour of the nodules present.

Ex. 95.—Examine the nodules on the roots of beans and peas (1) when the plants are a month old; (2) when they are just coming into flower; and (3) when the seeds are almost ripe. Cut sections of the nodules, and note the difference in the external and internal colour and texture of each at the different stages of growth of the plants.

Ex. 96.—Cut transverse and longitudinal sections of a bean nodule. Examine with a low power, and make drawings indicating the position and form of the various tissues. Then examine with a high power, and make detailed drawings of the several parts.

Ex. 97.—Cut transversely across a nodule of a bean root: dig out a small portion from the central part exposed, and crush it in a few drops of distilled water. Then take a small drop of the latter, and spread it over the surface of a clean cover-slip.

When dry float it, bacteria side downwards, on a very weak solution of gentian violet, and leave to stain all night.

Excellent stained specimens of the nodule organisms are obtained in this way.

Ex. 98.—Prepare the following solution:—

Mono-potassium phosphate (KH_2PO_4)	1.0 gr.
Magnesium phosphate . . .	.05 gr.
Distilled water.	500 c.c.

To one 100 c.c. add .5 gr. sodium succinate.
,, another 100 c.c. ,, 1 gr. glycerine.
,, ,, 100 c.c. ,, 1 gr. peptone.
,, ,, 100 c.c. ,, 2 gr. mannite.

Heat each to boiling, and filter: then pour 10 to 15 c.c. of each into sterile tubes, and sterilize on three successive days.

Inoculate a tube of each with organisms taken from the interior of nodules of pea, runner bean, red clover, and broad bean, with a sterilized knife and needle.

Incubate for fourteen to twenty-one days, and then examine a drop of the culture for "bacteroids" in a fresh state and stain with dilute gentian violet as in the previous experiment.

In which medium do the organisms grow least satisfactorily?

Ex. 99.—Grow the nodule organisms on Harrison and Barlow's wood-ashes-maltose agar.

Add 8 gr. of well-burnt wood ashes to 500 c.c. of distilled water, and boil for one minute: filter through two sheets of paper.

To 400 c.c. of the filtrate add:—

4 gr. of agar,
and 4 gr. of maltose.

Heat until dissolved: then filter, tube, and sterilize in the usual way, leaving some to solidify for agar slants.

Now isolate and prepare pure culture of the nodule organisms from beans, peas, red clover, and other leguminous plants.

Dig up the plant and wash the roots. Remove a clean nodule with forceps, taking care not to squeeze it too much, and immerse it for one minute in mercuric chloride solution :—

Mercuric chloride	.25 gr.
Hydrochloric acid, sp. g. 1.2 . .	.5 c.c.
Water	250 c.c.

Take it out with sterile forceps, and place it on blotting-paper to absorb the solution. Cut it open with a sterile knife : dig out with the flamed point a small portion of the bacteroidal tissue from the centre of the nodule, and transfer it to a heated watch-glass containing a drop or two of sterile water.

Inoculate a tube of melted agar with a loopful of the liquid on the watch-glass, pour into a Petri dish, and incubate at 20° C.

In two or three days note the white paraffin-like shining colonies ; examine and stain organisms from them.

Make streaks on agar slants.

Ex. 100.—Prepare wood-ash-maltose solution (Harrison and Barlow).

Take 8 grams of good, well-burnt wood ashes, and put them in a flask with 500 c.c. of distilled water : boil for a minute, and then filter.

To 400 c.c. of the filtrate, add 4 grams of maltose, and boil.

Then pour about 10 c.c. into a number of sterile tubes, and sterilize in the usual way.

In these tubes grow the nodule organisms obtained from nodules in the manner described in the previous experiment.

CHAPTER XIII.

BACTERIOLOGICAL EXAMINATION OF SOILS.

Comparison of the Fermentative Action of Soils.—The determination of the numbers of bacteria in soils of different fields has hitherto not given very reliable indications of the relative fertility of the latter. Somewhat better results have been obtained, however, by a comparison of the fermentative activity of the different soils when introduced in similar amounts into various nutritive solutions, and no doubt it is in the improvement of this kind of method of soil examination that our knowledge will be increased of the relationship between the fertility of the soil and the biological phenomena which occur within it.

What is required are reliable methods of determining the kinds and relative proportions of bacteria in the soil, and the work which each kind is capable of performing, rather than means of determining their total number.

Remy, Löhnis, Buhlert, Fickendey, and others have attempted to compare the bacteriological character of soils in respect of their power of decomposing proteins by introducing similar amounts of the different soils into peptone solutions, and estimating the amount of ammonia produced in a given time in each case. In a similar way the relative power of nitrification, denitrification, and nitrogen fixation possessed by various soils have been compared.

The work is at present of a tentative character: there is great difficulty of obtaining small samples of soil which are strictly comparable and representative of the whole field or garden, and further research is necessary to ascertain the most suitable solutions to be used in the work.

It is hoped, however, that investigations along these lines may ultimately lead to the discovery of a rapid means of comparing the fertility of soils.

Ex. 101.—Dig up a block of soil with a spade: break it, and from the untouched portion remove about a lb. with a knife or trowel sterilized in the field with a spirit flame. Store the sample in a wide-mouthed sterilized bottle.

Take 300 gr. of the soil, and add it to a flask containing 300 c.c. of sterile tap water: shake the contents for five minutes.

(i) **Test the putrefactive power or peptone-destroying power of the soil.**

Prepare a $1\frac{1}{2}$ per cent. solution of peptone in tap water, and sterilize it. Place 10 c.c. of this solution in a test-tube, and add to it by means of a wide-bored pipette 5 c.c. of the above turbid soil and water mixture.

Incubate at 25° C. for four days. Then pour the contents of the tube into a flask to which is added 1 or 2 grams of calcined magnesia, and a drop or two of paraffin, to prevent subsequent frothing of the liquid.

Distil off the ammonia from the compound produced by the action of the soil bacteria on the peptone into $\frac{1}{20}$ normal sulphuric acid.

(ii) **Test the urea-splitting power of the soil.**

Take 10 c.c. of Beijerinck's urea solution (p. 225). Add to it 10 c.c. of the above soil and water mixture, and incubate at 22° C. or three days.

Estimate the amount of ammonium carbonate produced by titration with normal hydrochloric acid.

(iii) **Find out if the soil possesses denitrifying power.**

Prepare the following solution :—

Grape sugar	1.5 gr.
Sodium nitrate	.3 gr.
Di-potassium phosphate . .	.1 gr.
Tap water	100 c.c.

Add 10 c.c. to test-tube, and sterilize. Then add 5 c.c. of the soil and water mixture, and incubate at 25° C.

Test for nitrate every day with diphenylamine and sulphuric acid as in Ex. 74 until the reaction disappears.

(iv) **Test the nitrifying power of the soil.**

Prepare the following solution :—

Ammonium sulphate	.4 gr.
Di-potassium phosphate (K_2HPO_4)	.2 gr.
Tap water	100 c.c.

Take 25 c.c. and add 1 gr. basic magnesium carbonate.

To this add 20 c.c. of the soil and water mixture, and incubate at 20° C.

After six weeks estimate the nitrate present

(*a*) by Schloesing's method, or

(*b*) by the colorimetric method of Grandval and Lajoux.

The latter depends on the principle that picric acid is formed when nitrates are treated with an excess of phenol-sulphuric acid, and this compound gives a strong yellow-coloured solution of ammonium picrate when dissolved in weak ammonia.

An empirical colorimetric scale is first prepared as follows :—

Dissolve 7.22 gr. of potassium nitrate in 1 litre of distilled water. Take 50 c.c. of this, and add it to 1 litre of water; from the latter measure out 100 c.c., and add it to a third litre of distilled water. 1 c.c. of the last dilution contains .005 gr. of nitrogen.

Add to watch-glasses or small beaker the following amounts of the third solution :—

1 c.c.	(which	corresponds	to .005	gr. of Nitrogen).
2 c.c.	"	"	.01	"
4 c.c.	"	"	.02	"
6 c.c.	"	"	.03	"
8 c.c.	"	"	.04	"
10 c.c.	"	"	.05	"
15 c.c.	"	"	.075	"
20 c.c.	"	"	.10	"

and evaporate all to dryness over a water bath.

Then pour over the dry matter on each glass 1 c.c. of a solution of

4 gr. phenol in
36 gr. sulphuric acid.

Dilute with 5 c.c. of water, and wash off each into separate narrow colorimetric cylinders with 15 c.c. of a 5 per cent. solution of ammonia, and fill up to the 50 c.c. mark.

Add to the series a cylinder of distilled water.

After preparation of the standard colour scale take the nitrified solution to be tested, and filter it. Wash the sediment in the filter with distilled water, and make up the volume of the filtrate to 100 or 150 c.c. Of this solution take 50 c.c., evaporate to dryness, and treat as indicated in the preparation of the scale.

Make up the solution of ammonium picrate obtained to 50 c.c., and place it in a cylinder alongside those of the scale for comparison of tint.

From the result calculate the amount of nitrate present in the nitrified solution.

(v) **Test the nitrogen-fixing power of the soil.**

Place the following solution in a large Erlenmeyer flask :—

Dextrose	4 gr.
Di-potassium phosphate . . .	.2 gr.
Sodium chloride	.2 gr.

Calcium carbonate	1 gr.
Tap water	200 c.c.

Add 20 c.c. of the soil and water mixture and incubate at 20° C.

After four or five weeks estimate the total amount of nitrogen in the flask by Kjeldahl's method, and deduct from it the average nitrogen content of 20 c.c. of the soil mixture which should be ascertained at the beginning of the experiment.

Ex. 102.—Repeat the above experiments with :—

1. a light sandy soil,
2. a peaty soil,
3. an ordinary arable soil,
4. a richly manured garden soil,

and compare the soils in respect of their powers of nitrification, denitrification, putrefaction, urea-decomposition and nitrogen-assimilation.

CHAPTER XIV.

FARMYARD MANURE.

1. **Nature and Composition of Farmyard Manure.**—A large amount of food consumed by a horse, cow, or other animal undergoes digestion in the stomach and intestines, being changed in the process into various chemical substances, which are absorbed into the blood stream, and carried to different parts of the body where growth is going on, or where the repair of tissues is needed. As a result of the vital activity of the organism, its substance is continually being broken down into waste products which must be removed or excreted from the body if healthy life is to be preserved. The fats and carbohydrates taken in are oxidised to water and carbon dioxide gas, the latter escaping into the air from the lungs in the act of breathing. The nitrogen of the proteins in the food and wearing tissues ultimately appears as urea and allied soluble nitrogenous substances which are excreted in the urine. The sulphur and phosphorus in nutrient materials are oxidized, and leave the body as alkaline sulphates and phosphates partly in the solid fæces and partly in the urine along with other dissolved salts. The undigested material passes along the intestinal canal, and is evacuated as fæces or dung.

Farmyard manure is composed mainly of three constituents, namely:—

(1) The fæces or dung, and,

(2) The urine,

of horses, cattle and other farm animals along with a certain amount of,

(3) litter used in bedding for the animals.

The dung in a fresh state contains from 70 to 80 per cent. of water, and is composed of the insoluble and undigested residue of the food with certain materials derived from the digestive juices of the intestinal canal. It is an exceedingly complex mixture, the chief substances present in it being woody fibre, undigested cellulose, fat and starch, mucus and bile pigments, together with certain decomposition products such as indole and skatole produced by putrefactive bacteria as well as fatty acids, alkaline soaps, and small amounts of magnesium and calcium phosphates. In the putrefactive and other fermentative changes occurring in the intestines considerable quantities of carbon dioxide, methane and hydrogen gases are produced, and these are found in freshly voided dung with free nitrogen derived from air which has been swallowed with the food.

The urine is a watery solution of chemical compounds derived from the digested food and from waste products arising in muscular and other tissues. It contains about 96 per cent. of water and 4 per cent. of dissolved materials. The most important substance present in it is *urea*, a nitrogenous crystalline compound originating from proteins absorbed by the blood, and in part apparently from leucine and tyrosine produced by tryptic digestion in the intestines. Urea is also a product of the disintegration or wear and tear going on in various organs. It amounts to about one half of the total solids of the urine. Other nitrogenous waste materials are uric and hippuric acids ; these are met with chiefly as sodium salts, sodium and potassium hippurate being especially

characteristic of the urine of herbivorous animals. About 1 per cent. of sodium chloride is found in urine also, and small amounts of acid sodium phosphate, phosphates of calcium and magnesium, and the sulphates of sodium and potassium.

The litter which is used to absorb the liquid excretions and provide a dry bed for horses, cattle and other animals kept under cover is usually the straw of some of the cereals ; but peat, bracken fern or dried leaves are used sometimes in place of straw. All these contain large amounts of cellulose with small quantities of other carbohydrates and nitrogenous bodies, as well as the ash of the vegetable tissues concerned.

Under ordinary conditions on a farm the fæces, urine and litter become more or less intimately mixed together to form farmyard manure. This mixture is very complicated in character and very varied in composition. The amount and nature of the food, and the kind and age of the animal consuming it, as well as the work done by the animal, influence the composition of the manure. The richer the food in proteins the richer will be the excreta in nitrogen : fattening animals extract and retain less of the food constituents than young growing animals, and so on. Careful experiments have shown that, as a rule, about 45 per cent. of the organic matter, 85 to 95 per cent. of the nitrogen, and 95 to 99 per cent. of the mineral constituents of the food are recovered in the fæces and dung where careful arrangements are made for the collection of the latter. The following analyses by Stoeckhardt indicate the chief differences in composition between the fæces and urine of the different farm animals.

FÆCES.

	Water.	Per cent. Nitrogen.	Phosphoric Acid.	Alkalis.
Horse .	76	·5	·35	·3
Cow .	84	·3	.25	.1
Pig .	80	.6	·45	·5
Sheep .	58	·75	.6	·3

URINE.

	Water.	Nitrogen.	Phosphoric Acid.	Alkalis.
Horse .	89	1.2	trace.	1.5
Cow .	92	.8	trace.	1.4
Pig .	97	·3	.13	.2
Sheep .	87	1.4	.05	2.0

The urine is rich in nitrogen and alkalis and poor in phosphates. Over 98 per cent. of the potash and soda of the food is found in the urine, while most of the phosphorus, magnesium, and lime compounds are obtained in the solid excreta.

The various substances in the liquid and solid excreta soon undergo chemical changes. The urine begins to ferment while it is in the stable, and further extensive decomposition occurs throughout the whole mass of the farmyard manure soon after it is placed on the heap, or allowed to accumulate in the yards. These changes are brought about by the agency of vast numbers of bacteria and fungi. Heat is developed mainly as the result of the oxidation of organic carbon compounds, and the heap is seen to " steam " in autumn or winter when the temperature is low enough to condense the water vapour which is given off. Much of the organic substance disappears as carbon dioxide and other gases, and the bulk of the manure is soon visibly decreased and its weight

reduced. Voelcker's analyses of a heap of farmyard manure composed of a mixture of the excreta of horses, cows, and pigs, with the usual straw litter, showed a loss of 20 per cent. of its weight when the straw was half rotted; at a later stage, when the heap was "fatty" or "cheesy" in texture, it was reduced 40 per cent., and when the fermentations or decompositions were practically complete the heap was found to have lost 50 to 60 per cent. of its original weight.

2. **Bacteria in Farmyard Manure.** — Normal urine when it is excreted is free from living organisms, but the solid fæces of all kinds of animals contains vast numbers of bacteria. Wüterich and Freudenreich examined the fresh dung of two cows fed on grass, and found that in one case the number present in a gram of dung was 10 and 12¼ millions respectively on two separate days, in the other the numbers were 1.8 and 4 millions on the same days. Later, after feeding the same animals with hay the number of the bacteria in the dung rose in the case of one cow to 187 millions, and in the other to 387 millions per gram. The numbers found by Savage in the dung of three horses examined was from 1½ to 2 millions, in four cows from 1 to 10 millions, and in three pigs examined the solid excreta contained 10 to 100 millions per gram. The most characteristic bacterium present in especially large numbers in the intestines and in the fresh fæces of all kinds of animals is *Bact. coli*, of which there are several very slightly modified forms differing in their power of fermenting carbohydrates. Wüterich and Freudenreich found *Bact. coli* chiefly in the fæces of cows along with a smaller number of *Bs. subtilis* and *Bact. Schafferi*. Savage found many streptococci as well as *Bact. coli* in the fæces of

farm animals, together with the spores of *Bs. enteritidis sporogenes*, the latter being especially abundant in fresh pig dung. Heinick showed that the excreta from pigs often contains *Bact. lactis aerogenes*, as well as *B. coli*, and large numbers of the former organism have been found in the intestinal canal and fæces of other animals also.

The bacteria mentioned may be considered natural inhabitants of the digestive tract : others are introduced in the food, and under certain circumstances may live and increase in number within the animal, and be found in the dung. A number of species also gain access to the manure while it is in the stable and farmyard, and multiply rapidly in it, the mixture of liquid and solid excreta proving an excellent nutrient medium for them. Some of the kinds are introduced in the litter and in the fodder ; others are conveyed by the dust in the air and the water used about the premises.

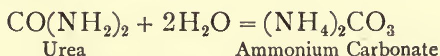
Sewerin isolated from horse dung thirty-two species or varieties, the majority of which in manure one month old were rod-shaped organisms : cocci were comparatively uncommon until the manure had been stored three months. In an old sample of stable manure Löhnis and Kunze found forms of *Proteus* and the uro-bacteria most abundant with large numbers of *Bact. fluorescens* (liquefying and non-liquefying forms) as well as *Bact. putidum*, *Bs. mesentericus*, *Bs. subtilis*, and *M. candicans* : many denitrifiers were isolated also.

3. While many of the chemical changes which take place in the dung heap are imperfectly understood, and will require much research before they are completely elucidated, some of the fermentations which occur have been extensively investigated and their nature

explained. Of these the following are the most important :—

(i) The ammoniacal fermentations of urea and allied nitrogenous compounds present in the liquid excreta.
(ii) The breaking down of the proteins in the putrefactive process (see pp. 100-103).
(iii) Nitrification and denitrification (see pp. 134-160).
(iv) The fermentation of cellulose.

(i) **The Ammoniacal Fermentation of Urine.**—Freshly excreted urine of man is slightly acid due to the presence in it of acid sodium phosphate, that of most herbivorous animals being neutral or slightly alkaline. However, on being exposed to the air for a few hours the urine of all animals becomes very strongly alkaline and develops an odour of ammonia, which is often prevalent in stables. The urea, uric and hippuric acids, also undergo fermentation with the production of ammonium carbonate, from which gaseous ammonia is set free. In the case of urea the change is one of hydrolysis, thus :—



$$\underset{\text{Urea}}{CO(NH_2)_2} + 2H_2O = \underset{\text{Ammonium Carbonate}}{(NH_4)_2CO_3}$$

A number of different species usually spoken of as "uro-bacteria" are capable of effecting this change. They are very widely distributed in the air, in dirty water, in dust and soil, and in manure heaps. Some of them are coccus forms while others are bacilli: the latter appear to prevail in soil. All are aerobic, and grow best at a temperature of 30° to 32° C. in 2 to 5 per cent. solutions of urea made slightly alkaline by adding .2 per cent. of

ammonium carbonate. A number of species form spores which resist the temperature of a 95° to 100° C. for an hour or more; others do not produce spores, and are easily destroyed by heating to 70° C. for a short time. Some of them grow an ordinary beef gelatine media, while others only develop on gelatine or agar media containing urea. They differ very considerably in their power of hydrolysing urea; *Urobacillus Pasteurii* under favourable conditions at 30° C. is able to decompose 3 gr. per litre per hour, while many of the *Urococci* are only able to ferment this amount in twenty-four hours. When grown on solid media containing urea a characteristic whitish ring or halo appears, consisting of minute powdery crystals of calcium carbonate mixed possibly with calcium phosphate.

The ammoniacal fermentation of urea by the uro-bacteria is due to the action of an enzyme, *urease*, which, according to Miquel is excreted into the surrounding liquid nutrient medium in which the organisms are cultivated. From such medium Miquel stated that an active solution of urease can be obtained by filtration through a porcelain filter, but Beijerinck, Moll, Leube, and others have not been able to confirm this. Beijerinck has shown that in the passage of a liquid culture of the uro-bacteria through filter paper many of the organisms are kept back and the filtrate has little fermentative action on urea, which would not be the case if the urease was in solution. A precipitate can be obtained by the addition of absolute alcohol to a ten to fourteen days old culture, which when dried at 30° to 35° C. and added to a solution of urea sets up an energetic ammoniacal fermentation at 48° to 50° C., specially in the presence of small amounts of glycerine and

saccharose. Possibly the urease may diffuse from dead cells, but it is most active as an intracellular enzyme of the living bacteria.

Comparatively little is known of the bacterial decomposition of uric and hippuric acids. The nitrogen in both appears to become changed into ammonium compounds, but the stages of the process and the organisms connected in it are very imperfectly known. In some experiments carried out by F. and L. Sestini, a solution of uric acid infected with decomposing urine and exposed to the air was changed into ammonium bicarbonate and carbon dioxide, and Gérard showed later that some organisms hydrolyse the compound into urea and tartronic acid, the former being acted upon later by the uro-bacteria and changed into ammonium carbonate.

Hippuric acid can be converted by acids and alkalis into its constituents benzoic acid and glycocoll (amino-acetic acid), and Van Tieghem states that the same change can be induced by *Urococcus ureæ.* Burri considers that calcium hippurate is fermented by certain bacteria with the production of ammonium carbonate, and Schellmann isolated a number of organisms from sewage and soil which broke down hippuric acid and glycocoll to compounds of ammonia, but not much is known of their morphology or relationship.

Micrococcus ureæ.—Cohn (*M. Van Tieghemi*, Miq.) is one of the most prevalent of the urea bacteria in the air, water, soil, and decomposing urine. It is a small coccus, .8 to 1.5 mm. in diameter, met with usually singly or in pairs and tetrads, never in chains. It does not form spores. Unlike most of the allied uro-bacteria, it grows on ordinary nutrient media containing no urea.

Gelatine.—The surface colonies on gelatine are non-liquefying,

white, round, semi-transparent, and somewhat slimy; some of them grow and reach a diameter of about 10 mm. in a week or ten days at 22° C. The "stab" is threadlike, white, and raised at the surface.

Urea gelatine (p. 224).—On this medium the colonies are surrounded by a ring of small crystals.

Agar.—Surface colonies are white with a somewhat pearly iridescence.

Bouillon.—This medium is rendered turbid in a day or two and a precipitate is produced.

Urea bouillon.—It grows rapidly in 2 per cent. urea bouillon, and the urea soon disappears.

M. ureæ liquefaciens, Flügge, is a uro-coccus, 1.25 to 2 μ in diameter, single, or three to ten cells in a chain. On gelatine its colonies are round, dark grey at first, yellowish-brown later, with a dark centre and wavy border. The "stab" liquefies in funnel-shape, the liquefied part becoming turbid, and deposits a yellowish-white precipitate.

Urococcus Dowdeswelli, Miquel, is an aerobic organism common in air, water, and the soil, oval, 2 to 3 μ long and 1 μ broad, usually met with singly or in pairs, but may grow into short chains. It does not form spores.

Gelatine.—The surface colonies are non-liquefying at first, later yellow; the "stab" is nail-like with a hemispherical head.

Agar.—On agar its colonies are yellow and soon extend to considerable size.

Potato.—Yellow growth.

Bouillon, becomes turbid with yellow deposit.

Urosarcina Hansenii, Miquel, is a coccus occurring in tetrads, or irregular heaps.

Gelatine.—The surface colonies are non-liquefying, small and yellow.

Bouillon, is rendered only slightly or not at all turbid, but a yellow sandy precipitate appears in a few days.

Urobacillus Miquelii, Beijk.—A motile bacillus 3 to 4 μ long, 1 μ broad, sometimes in pairs; no spores are produced. It was isolated from soil and grows in ordinary media. It is frequent in soil and has little urea fermenting power.

Gelatine.—The colonies are yellowish-white or sometimes pale rose, of irregular branched outline, closely resembling those of *B. Zopfii.* After growing for a time on gelatine it loses its power of hydrolysing urea, but becomes more energetic in liquefying gelatine.

Urobacillus Leubei, Beijk.—A motile red-shaped organism isolated from soil. It is 3 to 5 μ long, 1.5 μ thick, and forms oval spores, .8 to 1 μ long: some of the spore-bearing cells are occasionally *Clostridium*-like. It grows on ordinary gelatine media.

Gelatine.—The spore-bearing surface colonies are 2 to 3 μ in diameter, dull, yellow and thin. The colonies of organisms which have not produced spores are brighter and more transparent. The gelatine is not liquefied even in old cultures. On ammonium carbonate gelatine the colonies are much larger and closely resemble those of *U. Pasteurii*; the latter, however, does not grow on ordinary beef gelatine, so that doubtful colonies may be distinguished by transference to this medium.

Planosarcina ureæ, Beijk.—A motile sarcina found in garden soil. It occurs in the form of packets of 4 to 8 or more cells, each cell about .7 to 1.2 μ in diameter. It produces round spores .6 μ in diameter. The power of fermenting urea is not so marked as that of the other coccus forms. It grows on ordinary beef gelatine.

Gelatine.—The colonies are flat, non-liquefying, pasty and yellowish.

Urobacillus Pasteurii, Miq., is an aerobic rod-shaped organism, 2 to 5 μ long and 1 to $1\frac{1}{2}$ μ broad, sometimes in pairs or longer chains. It produces spores usually near the end of the cells and not infrequently appears as a *Clostridium* form. It is motile and is Gram positive. The optimum temperature

for growth is about 32° C. It will not grow on ordinary media, but thrives when provided with urea and ammonium carbonate. It is commonly present in manured soils, and is one of the most energetic of all uro-bacteria, being able to ferment large amounts of urea in a short time.

Urea Gelatine (p. 224).—The surface colonies are round, moist, greyish-white, slow in growth, often not more than 3 mm. in diameter after a week. The centre after a time becomes somewhat concave and the medium partially liquefied. Deep colonies are at first small, yellowish or brownish in colour: they may come to the surface and grow out into a thin transparent film 1 μ or more across.

Urea bouillon (p. 223).—The liquid becomes slightly turbid and develops an odour of ammonia. No growth occurs on ordinary gelatine, potato or agar media, or in bouillon or milk.

Urobacillus Schutzenbergii, I., Miquel.—A motile, short, rod-shaped organism, without spores, 1 μ long, .3 to .5 μ thick, generally occurring in pairs.

Gelatine.—The surface colonies are round, semi-transparent, milky, and cup-shaped. The gelatine is liquefied.

Urea-gelatine. (p. 224).—On 2 per cent. urea-gelatine the colonies are 1 to 2 mm. in diameter, and surrounded by a halo of crystals; no liquefaction occurs.

Agar.—On agar the colonies are greenish white.

Urobacillus Schutzenbergii, II., Cambier, is a motile aerobic organism, 3 to 5 μ long, .6 μ thick, without spores.

Gelatine.—The colonies are white, and produce rapid liquefaction of the medium.

Agar.—The surface colonies are white, small, with a slightly pearly lustre.

Bouillon is rendered turbid, and remains so for some time, with little precipitate.

Urobacillus Duclauxii, Miquel.—A common uro-bacillus in river and canal water, and a facultative anaerobe. The cells are 2 to 10 μ long, .6 to .8 μ thick, and motile in alkaline media.

It forms oval spores, which resist a temperature of 95° for two hours. It only grows satisfactorily in media containing urea or ammonium carbonate, and at a somewhat high temperature, namely, about 40° C.

Urea-gelatine (p. 224).—On this medium the colonies are very small; no liquefaction occurs.

Bouillon containing ammonium carbonate becomes turbid and ropy, with an unpleasant odour.

Urobacillus Maddoxii, Miquel, is a motile bacillus, 3 to 6 μ long, 1 μ thick, isolated from river water and sewage. Oval spores are found. It grows very poorly in bouillon or on gelatine media; even where the latter contains urea the development is small, but crystal formation is recognizable. On ammoniacal agar plates it grows better; the colonies are thick and white.

Urobacillus Freudenreichii, Miquel.—A motile aerobic bacillus, 5 to 6 μ long and 1 μ thick; it forms spores, and was isolated by Miquel from soil, cow manure, and river water.

Urea gelatine (p. 224).—The colonies are round, white, non-liquefying, and surrounded by a ring of crystals.

Bouillon.—In two to three days the medium becomes turbid, later the cloudiness disappears, and a white precipitate is thrown down.

Ex. 103.—Prepare the following urea-bouillon:—

Water	100 c.c.
Lemco's beef extract . .	5 gr.
Witte's peptone . . .	1.0 gr.
Urea	10.0 gr.

Boil; cool. The medium should be slightly alkaline; if not, add a drop or two of ammonium carbonate solution. Place in a 250 c.c. flask and infect with a gram of garden soil, and incubate at 20° to 30° C. for ten days.

(*a*) Examine a drop of the fermenting medium from day to day, and determine if cocci or bacilli are present. Stain with gentian violet. At first *Urococci* or *Planosarcina* may be present,

but later an almost pure culture of *Urobacillus Pasteurii* is obtained, the large amount of urea (10 per cent.) inhibiting the growth of most of the other organisms met with in the soil.

(*b*) Transfer a drop to a fresh flask of the medium, incubate for ten days at 30° C., and examine as before.

(*c*) Repeat the above, using garden soil which has been heated to 90° C. for one hour instead of a fresh sample.

Ex. 104.—Prepare 250 c.c. urea-bouillon as above; infect with garden soil and incubate at 22° C.

Remove 20 c.c. of the solution with a pipette, and titrate with normal hydrochloric acid. Repeat the titration of 20 c.c. drawn from the flask every three days, and note the increasing amount of acid needed for neutralization. The increased alkalinity is due to the formation of ammonium carbonate. 22 c.c. of normal HCl neutralizes about 1 gram of ammonium carbonate.

Ex. 105.—Expose some urine in a cool, shady place to the open air in a shallow vessel. Examine, after two or three days, for bacteria present; stain with gentian violet.

Ex. 106.—Prepare (i) 2 per cent. urea-gelatine; (ii) 2 per cent. urea-agar.

(i)	Water	1000 c.c.
	Lemco's extract (or beef broth)	5 gr.
	Witte's peptone . . .	10 gr.
	Gelatine	100 gr.
	Urea, ...	20 gr.

(ii) Same as above, using 15 gr. agar in place of the gelatine. If acid neutralize with ammonium carbonate; sterilize and tube as usual.

(*a*) Inoculate a tube of each with a drop of the liquid from Exs. 103 and 105. Pour into Petri dish and incubate—

(i) The gelatine medium at 22° C.

(ii) The agar medium at 30° C.

(*b*) Examine the organisms from the several kinds of colonies on the plates.

(*c*) Observe the urea fermenting colonies which are surrounded with small white crystals of calcium carbonate.

(*d*) Transfer portions of the different colonies into flasks of the following nutrient medium suggested by Beijerinck :—

Water	1000 c.c.
Sodium acetate	10 gr.
Mono-potassium phosphate (KH_2PO_4)	25 gr.
Urea	50 gr.

Incubate each at 22° or 30° C.

Note and compare the production of ammonium carbonate by titrating 10 c.c. of the solution from the different flasks at intervals of one to three days.

Ex. 107.—Add about 2 grams of garden soil or decomposing urine to the following solution :—

Di-potassium phosphate . .	.25 gr.
Ammonium sulphate . . .	1.0 gr.
Urea	5.0 gr.
Tap water	100 c.c.

Incubate at 3° C. ; after forty-eight hours the solution contains urea-splitting organisms chiefly.

With a drop of this culture inoculate tubes of the following protein-free agar medium suggested by Söhngen.

Calcium malate	5.0 gr.
Di-potassium phosphate . .	.5 gr.
Urea	10 gr.
Agar	15 gr.
Tap water	1000 c.c.

Repeat the observations (*b*) to (*d*) of previous experiment on the colonies which appear on this medium.

(ii) **Putrefaction in the Manure Heap.**—Although a large amount of the protein substances present in the food of an animal is digested and absorbed from the intestinal tract, some remains and passes out in the fæces, either

little changed or broken down to a considerable extent into simple compounds.

The putrefactive decomposition which begins in the intestines of the animal is continued in the manure heap: its nature and the organisms which carry out the work have been discussed in a previous chapter (pp. 100-114), which should be referred to again in this connection.

Ex. 108.—Take 5 or 10 grams of fresh manure of the horse, cow, and pig. Shake each portion in a flask containing 200 c.c. of sterile water: transfer 10 c.c. of the turbid liquid to another similar flask containing 200 c.c. of sterile water. From this second flask inoculate a tube of gelatine or agar medium and transfer to Petri dish. Incubate at 20° to 25° C.: note the kind of colonies obtained and examine the organisms producing these. Prepare pure cultures of those which appear to be distinct. Cultivate in various media and endeavour to determine their species, comparing with authentic pure cultures of *B. coli*, *B. aerogenes*, *Proteus* sp., *B. fluorescens*, etc.

Ex. 109.—Repeat the above experiment, using old decayed manure.

(iii) **Nitrification and Denitrification.**—The nature of both these processes and the bacteria to whose activity they are due have already been treated in detail previously (see pp. 135-168). Under ordinary conditions little nitrification occurs in the manure heap except perhaps in the outer layers, and it is not until the manure has become incorporated with the soil that its nitrogenous compounds become rapidly changed into nitrates.

(iv) **The Fermentation of Cellulose.**—The solid fabric of the body of a plant consists chiefly of cell-walls, which in the main are composed of a carbohydrate termed cellulose, having the empirical formula $C_6H_{10}O_5$. It is

a compound insoluble in water or weak acids and alkalis, and under certain conditions can be hydrolysed into a hexose sugar. Through mixture or combination with other substances the cellulose of young cells becomes greatly modified in its chemical and physical properties, and a series of "compound celluloses" are formed. Thus we have the *pectocelluloses* of cotton and flax fibres, and the parenchymatous tissues in the flesh of turnips and mangels which contain cellulose mixed or combined with pectose: the *adipocelluloses* forming the cell-walls of cork containing a waxy or fatty material suberin, and the *lignocelluloses* of timber and lignified vessels and fibres in the vascular tissues of all the higher plants. Paper is composed of one or other of these celluloses obtained from wood, straw or cotton and linen fibre.

After the death of the plants, many of the celluloses in them resist decomposition for a time, but sooner or later they are hydrolysed to soluble carbohydrates, which under aerobic conditions are finally oxidized to carbon dioxide and water, the latter ultimately finding their way back into the air and soil from which they were originally derived. This breaking down of cellulose is carried out by certain bacteria and fungi, the end products of the process being carbon dioxide and water where free and abundant access of air is permitted. In situations where the air-supply is restricted, as in the mud of marshes and stagnant ponds, the cellulose is fermented by a number of anaerobic bacteria and methane, or marsh gas (CH_4), hydrogen and carbon dioxide are produced together with small amounts of butyric and other fatty acids. The gases are seen to rise in bubbles when the mud and rotting vegetation at the bottom of a pond is stirred with a pole or stick, and vessels completely filled with

nutrient solutions containing straw, paper or dead leaves in "suspension" soon begin to evolve methane after inoculation with a small amount of such mud.

Henneberg and Stohmann found that from 50 to 70 per cent. of the cellulose present in the hay, straw, and other food consumed by horses and cattle disappears in its passage through the alimentary canal of these animals and is not recovered in the fæces. The extent of the loss depends on the kind of cellulose, but even the resistent forms met with in paper and wood shavings are partially decomposed when taken into the bodies of farm animals. It was at first assumed that these compounds were digested by the enzymes of the stomach and intestines, but the experiments of Tappeiner and others have shown that the greater portion of the decomposition is due to the action of cellulose-fermenting bacteria existing in the first stomach of cattle and in the colon and other parts of the large intestines of herbivorous animals generally. The organisms break down the cellulose into methane, hydrogen, and carbon dioxide, and small amounts of other substances, which are used as food or excreted in the urine and fæces. Tappeiner showed that paper, straw, and other vegetable debris can be fermented by material taken from the stomach and intestines of cows and horses, the products obtained being the gases just mentioned, and small quantities of acetic and isobutyric acids.

In manure heaps fermentation of the cellulose of the litter and undigested residue of the food goes on and the bulk of the organic material rapidly diminishes. In the upper well-aerated parts of the heap complete oxidation occurs, and carbon dioxide and water are the end-products of the change; but in the interior where

anaerobic conditions prevail, methane, hydrogen, and carbon dioxide and other substances are formed. Deherain and Dupont made analyses of the gases within a large heap of mixed farmyard manure. Below are some of the results :—

August 24, 1899	Temp.	CO_2	CH_4	H	N	O
Top of the heap	71°	50	17.4	3.1	29.5	0
Middle	67°	68	23.9	7.4	.7	0
Bottom	63°	49	40.8	3.9	6.0	0
About a month later (Sept. 20 1899)						
Top of the heap	66°	42.7	52.4	0	9.8	1.1
Middle	65°	49.5	48.3	0	2.2	0
Bottom	52°	47.8	51.2	0	1.0	0

Van Tieghem, Hoppe-Seyler, and the earlier workers at cellulose fermentation, concluded that the chief organisms concerned in the process were *Bacillus amylobacter* and allied species of butyric bacteria. According to Van Senus, *Bs. amylobacter* works symbiotically with an aerobic species in the decomposition of cellulose. Certain it is that many of the butyric forms which stain a blue colour with iodine are present when the cellulose tissues of plants are undergoing fermentation and disorganization under anaerobic conditions, but further experiments are needed to determine the particular part which they play.

The isolation of two forms of bacteria capable of breaking down cellulose with the formation of methane and hydrogen was accomplished by Omeliansky in 1895. Pieces of filter paper and cotton wool were placed in flasks of nutrient solutions containing potassium phosphate, magnesium sulphate, ammonium phosphate and

chalk, and a small quantity of fresh horse dung or river mud was added. The cultures were kept at 35° C. for some months, and the gases evolved were collected at short intervals over mercury and analyzed. When fermentation was complete about 2 grams of filter paper were found to have yielded 552 c.c. of mixed gases, 191 c.c. being methane, and 361 c.c. carbon dioxide. At the same time, acetic and butyric acids were produced in the proportion of 9 molecules of the former to 1 of the latter. The bacteria giving rise to this type of fermentation were found to be anaerobic, rod-shaped bacilli, slightly bent and about 5 μ long and .4 μ thick. They form spores 1 μ in diameter at the ends of the cells, the latter then resembling miniature drumsticks (Fig. 37).

Fig. 37.—Organism which gives rise to methane fermentation of cellulose (×750).

Omeliansky discovered also that in river mud and fresh horse dung another very closely allied organism was present, which in pure cultures ferments cellulose with the production of carbon dioxide and hydrogen, no methane being formed. Under the action of this bacterium about 3.5 grams of cellulose gave 810 c.c. of gas, 154.3 c.c. of which were hydrogen and the rest carbon dioxide. Acetic and butyric acids were produced as in the methane fermentation but in a different proportion, there being 1.7 molecules of the former to 1 of the latter. The organism responsible for the hydrogen fermentation of cellulose is similar in form, but somewhat larger than that which gives rise to methane, being 4 to 8 μ (or sometimes 10 to 15) long, and .5 μ thick;

its spores are round and 1.5 μ in diameter, produced at the end of the cells as in the allied species. Neither kind stain blue with iodine and are therefore different in this respect from *Bs. amylobacter*. They have not yet been cultivated on solid media and to separate them is a tedious operation.

Ex. 110.—Prepare the following solution:—

Water	1000 c.c.
Mono-potassium phosphate (KH_2PO_4)	1 gr.
Ammonium phosphate . . .	1 gr.
Magnesium sulphate . . .	.5 gr.
Sodium chloride . .	a small crystal.

Pour into a 250 c.c. flask, and add 5 grams of calcium carbonate. Introduce about 3 grams of filter paper or blotting paper cut into small pieces. Then add a small piece of fresh horse dung or some mud from the bottom of an old pond. Fill the flask completely with the solution, and insert a cork through which passes a bent glass tube, arranged so as to collect any gas given off. Keep in a warm room. Observe the evolution of bubbles of gas which begins in three or four weeks in an inverted test-tube over mercury.

(1) When the test-tube is nearly full force into it with a syringe or pipette, some concentrated caustic potash solution. CO_2 is absorbed by the latter. Notice the diminution in the volume of the gas.

(2) Place the thumb over end of the tube, turn the tube mouth upwards and apply a light; CH_4 burns.

(3) Remove a bit of the filter paper after two or three months, and tear off small pieces with needles; mount on a glass slide or cover slip, and examine for bacteria. Stain with carbol fuchsin and methylene blue.

CHAPTER XV.

MILK: ITS ORIGIN AND COMPOSITION.

1. **Secretion of Milk.**—The milk of animals is a peculiar secretion manufactured in special glands known as mammæ. The udder of the cow consists of such glands, with which are associated four teats. Each teat has a small single orifice through which the milk escapes when pressure is put upon it as in the act of suckling or milking. Just inside the orifice of the teat and for some distance inwards is an open cavity, the *lactiferous sinus* or "milk-cistern," in which the secretion is stored when the animal is "in milk." From the reservoir there branch off into the substance of the udder a series of tubular channels or *lactiferous ducts*, irregular in diameter and distribution, but becoming smaller and smaller as they are traced backwards. The finest branches, of which there are thousands, ultimately end in exceedingly small pouch-like structures termed *alveoli* (Fig. 38). It is in these that the actual manufacture of milk takes place. Each alveolus is about $\frac{1}{200}$ of an inch in diameter and consists of a hollow cavity lined with a single layer

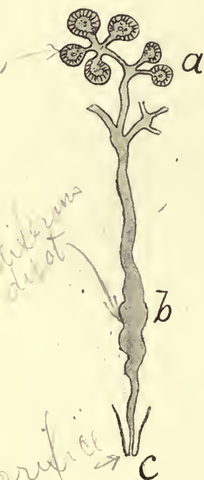


Fig. 38.—Diagram illustrating origin of milk in udder of cow. *a* Alveoli in which secretion takes place; *b* lactiferous duct; *c* orifice of teat.

of secreting cells. When the gland is inactive and not secreting milk these cells are cubical or somewhat flattened, and contain granular protoplasm with a single oval nucleus (Fig. 39). When milk is being produced they undergo peculiar changes. They increase in length very considerably and extend into the cavity of the alveoli, thus reducing its empty space. Each cell may now possess two or more nuclei, the basal one normal in form and size, the other near the free end of the cell being more or less degenerate and irregular in outline and structure. Within the cells are produced numbers of globules of oil or fat, as well as other materials, and from them water, salts of various kinds, sugar, and probably some of the oil, is discharged into the cavity of the alveolus and tend to fill it. Sooner or later the elevated portions of the secreting cells, with their degenerate nuclei, protoplasmic contents and oil, are cast off into the cavity of the alveoli and float in the liquid already secreted into it, the whole mixture forming what is known as *milk*. The detached solid parts of the cells

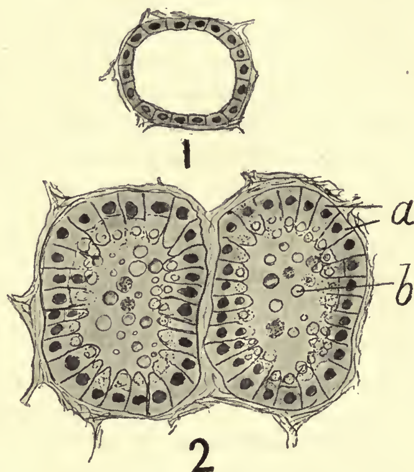


Fig. 39.—Transverse sections of alveoli of milk glands of cow.
1. Inactive alveolus.
2. Alveoli during milk secretion; *a* secreting epithelium; *b* fat globule in cavity of alveolus. (×300).

undergo disintegration and partial solution in the more liquid portion of the secretion. From the above it will be gathered that milk is not merely a secretion like saliva or gastric juice exuded from the secreting cells, but consists of a certain amount of a liquid secretion, together with solid portions of the secreting cells themselves, which have undergone transformation and a kind of fatty degeneration and then been cast off into the previously accumulated liquid in the alveoli. The materials from which the secreting cells manufacture milk are derived through the lymph from the blood stream which circulates in an extensive network of capillary blood vessels surrounding the alveoli. Few if any materials are passed on into the milk from the blood stream direct without transformation, but white blood corpuscles or leucocytes often make their way between the secreting cells from the blood into the milk, in which they may be easily recognized.

2. **Composition of Milk.**—From the foregoing account of the formation of milk it may be inferred that its composition must necessarily be complex. It contains a large number of organic compounds both solid and in solution. When freshly drawn it possesses a so-called *amphoteric reaction*, *i.e.* it is acid to blue litmus and alkaline to turmeric paper, a peculiarity associated with the presence in it of acid phosphates, citrates and certain proteins. The chief constituents present in cows' milk are water, proteins, fats, sugar and mineral materials, with a very minute trace of colouring substance, the following representing the general average composition according to Richmond :—

Water	87.10
Proteins	3.40

Fat	3·90
Milk sugar	4·75
Mineral matter . . .	·75

Freshly drawn milk also contains a small quantity of carbon dioxide gas and takes up oxygen and nitrogen of the air during the process of milking. The fat and some of proteins are suspended in the water, the rest being in solution. The term *milk-serum* is applied to the liquid portion of the milk in which the fat and solid materials float. It will be useful to deal with each of these constituents separately.

(i) **Proteins.**—These are highly complex organic substances, containing carbon, hydrogen, oxygen, and nitrogen with a small amount of phosphorus and sulphur. There are several kinds present in milk, the following being the chief:—*Caseinogen*, *lact-albumin*, with traces of *lacto-globulin*, and certain enzymes, the nature of which is imperfectly known. More than 80 per cent. of the proteins in milk is in the form of a complex phosphoprotein, which we shall speak of as *caseinogen*. It is not present in the blood of the cow, but is a product manufactured in and by the secreting cells of the mammary glands: it is in combination with calcium as explained below.

Unfortunately the term *casein* has been and is still used for this compound as well as for the two curds thrown down when acids or rennet are added respectively to milk, although these curdy substances are quite different from each other and from the original protein in milk.

While it is not possible in the present state of knowledge to define exactly the relationship of these "caseins" to each other, to prevent needless confusion it is

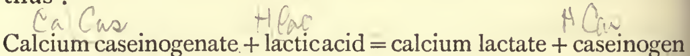
essential that three separate terms with limited connotation should be used for them.

The protein caseinogen is an acid body, capable of uniting with soda, potash, and lime to form salts, those of the two alkalis being soluble in water. The condition in which it exists in milk is a matter of dispute, but the most reliable evidence seems to support the view that it is combined with calcium as *calcium caseinogenate* along with a certain amount of calcium phosphate.

From the fact that caseinogen or its compounds do not pass through a porcelain filter, and can be collected as a slime by centrifuging milk, it would appear that these bodies are present in the form of minute particles suspended in the milk serum.

Calcium caseinogenate can be thrown down as a precipitate by saturating milk with sodium chloride, magnesium sulphate, or other agents used for "salting out" proteins (p. 86).

When lactic, acetic, or other acids are added to milk, a reaction takes place, which may be represented thus :—



Calcium caseinogenate + lactic acid = calcium lactate + caseinogen

the free caseinogen being a substance insoluble in water, appears as a precipitate which we may call *acid-curd.* Such a curd, of course, is simpler than the caseinogenate from which it was derived ; in a pure state it contains no calcium, although as usually obtained it has entangled within it some of the milk-serum and its dissolved calcium phosphate, as well as the fat-globules of the milk.

Rennet contains a coagulating enzyme, *rennin*, which, when added to warm unboiled milk, precipitates a

coagulum or curd, named by some authorities *casein*, but often spoken of as *paracasein* : it may also be termed *rennet-curd*. The exact nature of the action is not thoroughly understood, but the coagulation apparently takes place in three distinct stages. The first action of the rennet is to change the caseinogen into a soluble body, which we may name "*soluble casein*" ; almost coincident with this, is the production of soluble calcium compounds ; the latter then throw down the casein as a dense coagulum which differs from the curd produced by acids in containing practically all the calcium phosphate of the milk. In it is entangled much of the fat originally diffused through the milk. In the absence of soluble calcium compounds, the "soluble casein" remains in solution or suspension, giving no visible evidence of its existence.

Soluble calcium salts do not precipitate a curd from fresh unrenneted milk, nor will rennet coagulate milk, from which the calcium salts have been removed by the action of ammonium oxalate until a soluble calcium salt is added.

As already indicated, caseinogen behaves towards the alkalis soda and potash as an acid body, uniting with them to form salts which dissolve readily in water ; these salts, although they are decomposed by acids with the precipitates of caseinogen, are not coagulated by rennet.

The following summary of the names in use for the phosphoprotein of milk and its derivatives may be useful :—

(1) In milk *calcium caseinogenate* (often loosely termed *casein*) is present.

(2) Acids precipitate free *caseinogen* (sometimes called *casein*).

(3) Rennet changes caseinogen into a soluble body, "*soluble casein*" (sometimes termed *paracasein*), which remains in solution if no soluble calcium salts are present.

Soluble calcium salts precipitate it as *casein* (or *paracasein*).

Lact-albumin is another protein of milk, but, unlike caseinogen, it coagulates on heating and can be obtained as a slimy substance by boiling the residual "whey" after the curd thrown down on the addition of an acid has been removed by filtration. The skim formed on the surface of fresh milk when it is boiled consists of coagulated lact-albumin with a certain amount of dried caseinogen.

(ii) **Fats.**—These consist of mixtures of the common solid animal fats, palmitin and stearin (compounds of glycerine with palmitic and stearic acids), with variable proportions of olein and other liquid fats, compounds of glycerine with oleic, butyric, caproic and other acids (see p. 78). The fats are present in the form of small spherical globules of variable sizes, the diameter of some being as high as .01 mm. while in others it is as low as .0016 mm. Like the caseinogen they do not appear to be merely collected from the blood-stream as such and transferred to the milk, but are formed by the chemical activity of the secreting cells from proteins and possibly from fats supplied to the gland.

(iii) **Milk Sugar.**—Milk sugar or lactose is present to the amount of 4 to 5 per cent. in solution in the milk and can be obtained by evaporating whey. It is a crystalline material with a moderately sweet taste, and is easily changed into lactic acid by many species of micro-organisms. Like the other constituents of milk it is

formed in and by the secreting cells of the mammary gland and does not exist in the blood of the animal.

(iv) **The inorganic salts or mineral constituents** of milk are many and variable, the chief being calcium phosphate. Without the presence of soluble calcium compounds, rennet cannot precipitate a "curd." Phosphates of potassium and magnesium are also present, as well as chlorides of sodium and potassium, iron, silica, and traces of other materials. In fresh milk there are considerable proportions of compounds of citric and other organic acids.

(v) **Gases of Milk**.—Milk drawn fresh from the cow contains up to 5 or 6 per cent. of its volume of gases, the chief of which are carbon dioxide, oxygen, and nitrogen, the former being in largest amount.

Ex. 111.—(*a*) Place a drop of milk on a slide and cover with a thin slip.

Examine with a $\frac{2}{3}$ in., $\frac{1}{6}$ in., and $\frac{1}{12}$ in. objective. Note the form and size of the fat globules. Measure (Ex. 10) and make drawings of them.

If possible examine and compare the fat globules of Jersey, Shorthorn and Ayrshire or Dutch cows.

(*b*) Add a drop of osmic acid solution to a drop of the milk and examine again as above: note the black colour of the fat globules.

Ex. 112.—Dip a piece of blue or neutral litmus paper into fresh milk and notice reddening or acid reaction. Dip a piece of turmeric paper into it and note.

Take 50 c.c. of fresh milk and add to it 2 c.c. of a 2 per cent. solution of phenol-phthalein in alcohol. Run in slowly from a burette a decinormal solution of caustic soda; stir and note how many c.c. are needed before a permanent pink colour is produced.

Ex. 113.—(*a*) Add a few drops of dilute hydrochloric acid to some fresh milk.

(*b*) Add 1 c.c. of commercial "rennet" to 10 of water: then pour 1 c.c. of the mixture into 10 of milk: casein is precipitated.

Note the difference in the texture of the curds obtained in the two cases.

Ex. 114.—Heat 20 c.c. of fresh milk in a wide test-tube to 35 or 40° C. and add to it 5 c.c. of a weak solution of commercial rennet (1 in 10 of water).

Note the formation of curd or precipitation of casein in a minute or less.

Ex. 115.—Try the previous experiment, using boiled rennet extract.

Ex. 116.—Take 50 c.c. of fresh milk in a flask warmed to 40° C. and add a few drops of acetic acid.

Filter off the curd and dissolve :—

(*a*) One part of it in a weak solution of caustic soda;

(*b*) another part in lime water.

Add a dilute solution of rennet to both and keep them at 40° C.: in which of them is a curd reproduced?

Ex. 117.—To some fresh milk in a test-tube add one or two drops of a saturated solution of ammonium oxalate: then add a few drops of a solution of rennet: keep at 40° C. for an hour and note absence of coagulation. Now add a few drops of a 2 per cent. solution of calcium chloride: coagulation occurs rapidly.

Ex. 118.—(*a*) To 20 c.c. of milk add 10 c.c. of water: heat to 60 to 70° C. and add a few drops of 20 per cent. acetic acid.

Caseinogen is precipitated.

Filter twice.

(*b*) Test a portion of the filtrate for lact-albumin by Millon's reagent (Ex. 45).

(*c*) Boil the rest and note the coagulation of the lact-albumin.

(*d*) Filter and test the filtrate for sugar. Add a few drops of copper sulphate solution and strong caustic potash until a blue clear solution is obtained and then boil: a yellow precipitate indicates the presence of sugar.

3. **Colostrum.**—This is the term applied to the secretion which is produced in the alveoli of the udder just before and for a few days after parturition of the animal. It is a yellow opaque liquid, alkaline in reaction, and sometimes contains traces of blood. On heating it becomes coagulated. A microscopic examination reveals the presence in it of a number of colostrum corpuscles from .005 to .025 mm. in diameter, some of which closely resemble leucocytes or white blood corpuscles with irregular nuclei, at the same time exhibiting living amœboid movement. Many of them contain fat globules while others appear to be more or less disintegrated cells, free from fat and nuclear bodies (Fig. 40). The average composition is near the following—

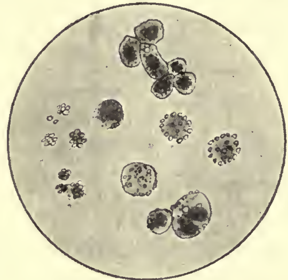


Fig. 40.—Colostrum corpuscles. (×500.)

Water	71.7 per cent.
Fat	3.4 per cent.
Caseinogen and other proteins	20.7 per cent.
Sugar	2.5 per cent.
Mineral matter . .	1.8 per cent.

On comparison with the average composition given on pp. 234-5 it is seen that colostrum differs very considerably from milk, in containing six or seven times as much proteins, less water, less sugar, and more mineral matter. The fat in it is of a more solid character than that of ordinary milk and the proteins consist of a little caseinogen with a larger proportion—12 to 14 per cent.—of albumin and globulin which are usually present in

very small amount only in normal milk. Dextrose as well as milk sugar is present. It curdles very slowly with acids and rennet: the change from the manufacture of colostrum to the formation of ordinary milk by the udder takes place gradually, but colostrum corpuscles do not disappear entirely for two or three weeks. The milk, although not unwholesome, is not generally used for dairy purposes until the lapse of three to five days after parturition, since cream containing it does not make good butter or cheese, and the milk cannot be so readily "separated." Before lactation the alveoli are found to consist of solid masses of cells, between which there are no alveolar cavities. Later the central cells of the alveoli with their nuclei and other contents are discharged, and according to some authorities become "colostrum corpuscles" alveolar cavities at the same time being established; others consider that the "colostrum corpuscles" are simply leucocytes which have absorbed fat as an amœba absorbs its food.

Ex. 119.—Obtain some fresh colostrum: place a drop of it on a slide and cover with a clean slip.

Examine with a $\frac{2}{3}$ in., $\frac{1}{6}$ in., and $\frac{1}{12}$ in. objective. Note the form and make drawings of the fat globules and colostrum corpuscles.

Spread a drop of colostrum on a cover-slip and allow it to dry: pass through flame and dip it in ether for three minutes and then in absolute alcohol for one minute, by means of which treatment the fat is removed from the film: dry and stain with Löffler's methylene blue, after which examine with $\frac{1}{6}$ and $\frac{1}{12}$ in. objective.

Make drawings of bacteria and other objects which have become stained a blue colour.

4. **The Enzymes of Milk.**—In 1897 Babcock and

Russell discovered that the proteins of milk underwent proteolytic disintegration even in the presence of ether, chloroform and other compounds which inhabit the growth of bacteria. Milk which contained on an average 20 per cent. of its proteins in a soluble form after being kept for three or four weeks in vessels to which antiseptics had been added to prevent bacterial action, was found to contain 38 per cent. of its proteins as albumoses and peptones. Milk which has been heated to a temperature sufficient to destroy enzymes does not undergo such changes. Subsequent investigation showed that the proteolytic decomposition of milk obtained and kept under aseptic conditions was due to the presence in it of a trypsin-like enzyme, to which the name of *galactase* has been given.

The amount in milk is very small, and although at first it was thought that it played a major part in the ripening processes of cheese, its influence in this direction is now known to be comparatively slight.

Traces of lipase, diastase, and other enzymes are said to occur in milk, but the evidence for the statement is not very convincing.

Positive results are readily obtained with tests for peroxidases (p. 115), but whether they are caused by galactase only or by other types of enzymes is not known with certainty.

Since the enzymes in milk are destroyed when the latter is heated for a short time to 80° C. the presence or absence of reaction with tincture of guaiacum or paraphenylendiamine and hydrogen peroxide affords a ready means of distinguishing boiled from fresh milk.

In some countries where regulations are enforced with a view of checking tuberculosis among farm stock, the

separated milk from public creameries must be pasteurized at a temperature not less than 80° C. before being returned to the farms for the feeding of calves and pigs; these tests assist in determining if the regulation has been carried out.

Ex. 120.—Put some lumps of gum guaiacum in a test-tube containing alcohol and boil for a short time. Filter the yellow solution, and gently add water until near the point at which a permanent milky emulsion is obtained.

(*a*) Add a small portion to some fresh milk, and then pour in a few drops of hydrogen peroxide solution. Slightly warm the milk: the production of a blue colour shows the presence of an oxidizing enzyme—peroxidase.

(*b*) Try the experiment with boiled milk.

Ex. 121.—Make a 2 per cent. solution of paraphenylendiamine "hydrochlorate."

(*a*) Take 10 c.c. of fresh unboiled milk, warm slightly, and add to it 10 or 12 drops of a 3 per cent. solution of hydrogen peroxide.

From a pipette slowly run into it down the side of the tube 1 c.c. of the paraphenylendiamine solution. Avoid shaking or mixing the contents, and note within five minutes the production of a blue colour showing the presence of peroxidase in the milk.

(*b*) Try the experiment with milk which has been heated for five minutes to 60°, 70°, 80°, 90°, and 100° C., and then cooled to about 50° or 55° C.

Determine the temperature at which the oxidizing enzymes are destroyed.

Milk contains a reducing enzyme, *i.e.* an enzyme which possesses the power of "reducing" or taking away oxygen from a compound in which oxygen is only in loose combination. Some *reductases* are direct reducers; others, however, such as that found in milk, can only effect reduction when a reducing agent, such as formal-

dehyde, is present also. Methylene blue and indigo-carmine, which lose their colour when reduced, are frequently used in testing solutions for these enzymes.

The reductase of milk is called *aldehyde catalase*. It is probably associated with the fat globules of the milk, reduction taking place more rapidly with strippings than with the poorer foremilk.

Ex. 122.—Prepare the following solution :—

Schardinger's reagent.

Saturated alcoholic methylene blue solution .	1 c.c.
Formalin	1 c.c.
Water	38 c.c.

(*a*) Add three drops to 10 c.c. of fresh milk, and pour in a small amount of melted or liquid paraffin to exclude oxygen. Keep the whole at 45° to 50° C., and note the disappearance of the blue colour in four hours or less.

(*b*) Try the experiment with (i) foremilk, (ii) midmilk, and (iii) strippings. Note the time taken to destroy the colour in each case.

5. **Action of Heat on Milk.**—When milk is boiled even for a few minutes it acquires a characteristic taste which is disliked, especially by children accustomed to the fresh liquid.

Its properties are considerably changed owing to alterations in the physical and chemical nature of several of its constituents.

The cooked flavour is acquired if a temperature of 160° F. is maintained for more than about ten minutes, and in much shorter time as the boiling point is approached. The objectionable taste, however, is removed to a large extent both from the cream and the

separated milk if the heated milk is passed through the separator.

Little change, if any, occurs in the chemical constitution of the fats, and cream can be heated to a higher temperature than milk before the cooked flavour is produced. The small groups or clusters of fat globules which are found in fresh milk are largely disintegrated into separate globules by heating, and the cream, which rises slowly, is thin.

The milk sugar is slightly oxidized, and acids are split off from it, the alkaline phosphates of the serum assisting the change; the milk becomes distinctly acid if it is boiled for three or four hours, and yellowish or brownish in colour, the altered tint, however, being more likely due to alteration in the proteins than in the sugar.

Very striking changes are made in the proteins of the milk by heating. The soluble albumin becomes coagulated and probably decomposed to some extent with the evolution of a small quantity of sulphuretted hydrogen at temperatures from 70° C. to 100° C. The pellicle or skin which forms when milk is heated in an open vessel consists of coagulated albumin and dried caseinogen, with fat and other constituents also.

The power of curdling by rennet and acids is very much reduced, a point of great importance to the cheese-maker. For the coagulation of the caseinogen more rennet is required and the curd is too crumbly and soft for the manufacture of hard pressed cheeses. The altered character of the caseinogen appears to be chiefly connected with the precipitation of insoluble calcium phosphate, and although some improvement in the curdling property can be made by the addition of dissolving agents

such as carbon dioxide, hydrochloric, and other acids, or by the addition of soluble calcium salts, no really characteristic Cheddar, Cheshire, or other hard cheese has yet been made from strongly heated milk.

As stated previously heating destroys the normal enzymes of milk. There is little doubt that the coagulation of the albumen, the changes in the solubility of the casein and calcium salts, and the destruction of the enzymes render the milk less digestible and reduces its nutritive qualities.

Ex. 123.—Mount a drop of fresh milk and one of the same sample after being boiled. Examine with a $\frac{2}{3}$ in. and $\frac{1}{6}$ in. objective and compare the arrangement of the fat globules in each.

Ex. 124.—Take 200 c.c. of fresh milk and divide it into two parts: boil one of these and then cool it. Taste both.

Add 10 c.c. of a weak solution of commercial rennet (5 of rennet in 50 of water) to the boiled and the unboiled samples in small beakers.

Note the time taken to curdle each: the exact point can be determined by scattering some pieces of charcoal scraped from the end of a burnt match on the surface of the milk and stirring with a glass rod: the floating particles stop at once when coagulation occurs.

Examine the curd produced: which is the firmest in texture?

CHAPTER XVI.

BACTERIA IN MILK AND THEIR SOURCES.

1. **Number and Source of Origin of Bacteria in Milk.**—In the last chapter the method of secretion of milk was discussed. In the present one it will be useful to deal with the contamination of milk by bacteria and the methods by which these organisms obtain access to it. As secreted by the alveoli of the healthy udder milk is quite free from bacteria, and it is possible to draw off from the interior of the udder a sterile sample by means of special apparatus. Moreover, it is very doubtful if bacteria, present in the blood or lymph, or causing local disease in the animal not involving the udder, ever gain access to the milk. However, where tubercular disease of the udder exists it is possible for the secretion to become infected with *Bacillus tuberculosis*. In various inflammatory processes in the substance of the udder and its milk ducts and ulceration in the teats, the milk may become contaminated with certain forms of streptococci, which are connected with the disease. Leucocytes, which are practically the same structures as pus-cells and from which the latter are derived, are probably met with in all normal healthy milk in small numbers as mentioned in the previous chapter; but when pus-cells are abundant, and especially if they are accompanied by many streptococci, the milk should be treated as diseased, and should not be used in the dairy or for domestic purposes. By some

authorities it is suspected that a relationship exists between certain types of streptococci of milk and scarlet fever, as well as sore throats and intestinal troubles, in persons consuming milk containing these forms of bacteria.

In addition to these pathogenic bacteria, which are only met with where the udder or parts of it is affected with some form of disease, milk contains certain kinds of non-pathogenic bacteria which have obtained an entrance to the lactiferous sinus or milk cistern and some of the ducts by way of the orifice in the teats. Generally speaking, they are most abundant in the milk accumulated in the lowest parts of the udder, and therefore obtained in quantity in the first milk drawn from it in the milking process. The number of species present in the milk in the udder are very few, the commonest being forms of streptococci, which are apparently innocuous and present in many cows which are quite healthy. They may be looked upon as more or less normal inhabitants of the milk ducts. Probably others obtain an entrance in the same way, but find the conditions unfavourable for their further growth and development, and consequently die out.

Many investigations have been made to determine the bacterial content of the milk which is drawn from healthy cows, the outsides of whose udders have been carefully brushed and washed and the milker's hands washed with antiseptic solutions to prevent the addition of bacteria to the milk from these sources. The milk first drawn is found invariably to contain considerable numbers of bacteria; Harrison obtained in one c.c. of such "foremilk" from 18,000 to over 50,000, Schultz as many as 97,000, while the investigations of Orr and Moore showed an average content of 3000 to 8000 per c.c.

As milking proceeds, the contaminated foremilk existing in the teat and milk cistern is soon removed, and the milk obtained later has very few bacteria in it. The "strippings," or last drawn milk, rarely contains more than 400 to 500 per c.c.

According to Swithinbank and Newman it is possible to draw quite sterile samples from the udders by means of a "milking tube" inserted through the teat after the "foremilk" has been milked out, if careful precautions are taken to sterilize the apparatus used and wash the udder and teats with an effective germicide before the operation.

On the other hand, it may be noted that Schultz, Freudenreich, Boekhout, De Vries, and others failed to obtain sterile milk from the udder of healthy cows, even where careful precautions were observed. There is little doubt that at the point of secretion, that is, in the fine alveoli of the mammary gland, milk is free from living organisms; the extent, however, of the invasion of the milk ducts in the lower portion of the udder is not clearly known. It probably depends to a large extent on the physiological condition of the cow and the kind of bacteria concerned; some organisms may be able to penetrate a considerable distance into the udder from the orifice of the teat, while others may be unable to exist or multiply at all under the conditions prevailing in the milk cistern and ducts.

As soon as milk is drawn from the udder it becomes liable to further pollution from various sources. Cows which are not carefully groomed have on their flanks, and on the udder to some extent, bits of dried dung, mud, and other materials, with which they are sure to come in contact when lying down in the cowshed,

yard or fields. During milking, portions of these are shed or rubbed off, and fall into the milk with hairs of the animal. An enormous number of organisms may be introduced in this manner, since old dried dung frequently contains more than 1,500,000,000 per gram, and many hundreds of thousands have been found attached to single hairs on the flanks of dirty cows. Orr obtained an average of 4752 colonies on agar plates exposed underneath the udder for two minutes during milking in the cowshed in winter. Where the udders and flanks of the cows had been brushed, 1752 colonies per plate were obtained, and in the case of four cows which were well-groomed and the udders washed and left slightly moist, similar plates left for the same period of time showed an average of only 230 colonies.

The milk from cows with brushed and washed udders was found by Russell and Orr to contain 330 to 472 per c.c., while the bacterial content of the milk of the mixed ungroomed herd averaged from 11,000 to 15,000 per c.c.

Many of the bacteria in milk are derived from the milkers. On many farms the milkers have no special garment or overall to slip on when milking is to be done, but milk in everyday clothing, which is frequently the reverse of clean. The filthy, disgusting habit of dipping the hands, often unwashed, into the milk when milking is going on is a very common practice which leads to bacterial contamination. The introduction of germs of disease brought from the homes of the milkers is likely to occur, and there is little doubt that scarlet fever, and possibly the bacteria of typhoid fever and diphtheria, have been transmitted to milk by the milker and other human beings concerned with the management of the cows and the handling of milk on the farm.

More attention should be given to the cleanliness of the milker, and his freedom from exposure to possible disease which can be transmitted through milk, than is generally the case.

Fig. 41.—Plate exposed one minute in cowshed before milking commenced.

Another source of contamination of milk is the floating matter of the air in the cowshed. This consists of dry particles of dung, dust from hay and other fodder, as well as many similar materials which are bearers of bacteria. Harrison found that the number of bacteria falling on a circular surface one foot in diameter (about equal to the opening of an ordinary milk pail) was from about 13,000 to 40,000 per minute in a cowshed where the cows had just been bedded. About an hour later much of the dust had settled, and the number deposited on a similar

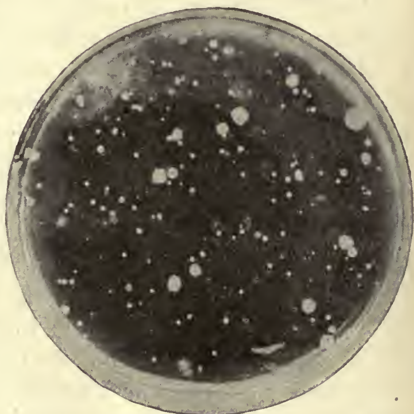


Fig. 42.—Plate exposed one minute while milking was being done.

area was found to be from about 450 to 2400 per minute. The use of milk-pails with narrower mouths than those in common use has been found to reduce contamination and improve the keeping quality of the milk (Figs. 41-44).

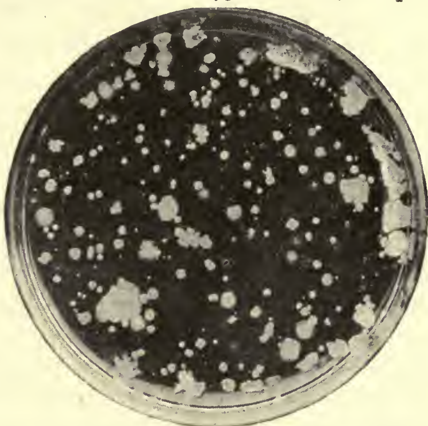


Fig. 43.—Plate exposed one minute in cowshed after giving the cows hay.

Flies also carry many objectionable bacteria from putrefying material upon which they settle, and are responsible for the introduction of these into milk in which they drown.

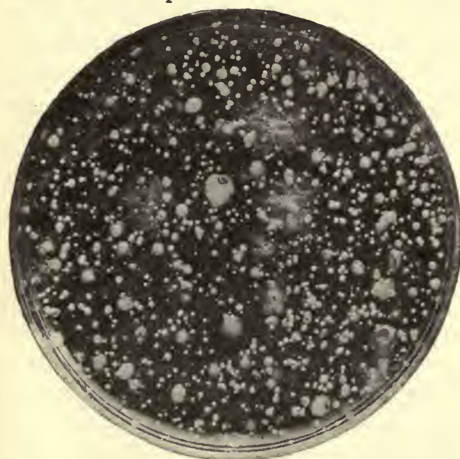


Fig. 44.—Plate exposed one minute after cowshed was brushed out.

Milking cans, pails, milk coolers, and other vessels on the farm, unless very carefully cleaned, are also fruitful sources of contamination. The cansorchurns in which the milk is despatched by rail are responsible for a very large share of the

contamination which occurs at the farm. These are often returned to the farmer without being cleaned properly, often with small amounts of sour milk in them, the latter sometimes containing as many as 300,000,000 per cubic centimetre. Even where steam had been used to clean the cans before being returned washings with 100 c.c. of sterile water were proved to contain from 400 to 1800 bacteria per c.c. Both Stocking and Orr have shown also that the use of milking machines increases the bacterial contamination very considerably: this is due to the difficulty of thoroughly cleaning the connecting tubes and also to the sucking in of dust laden air when the cusps fall off the teats of the animals.

The water to which the cows have access, and which is used for washing and rinsing the cans, churns, and other vessels in the cowshed and dairy, often contain bacteria, which find their way into the milk.

The cow, the milker, the air of the cowshed, flies, water supply, and imperfectly cleaned farm dairy utensils, are the main sources from which milk becomes contaminated with bacteria on the farm, and, without doubt, it is here that the chief troublesome forms become associated with it.

As already indicated, the number found in any particular sample will depend upon the amount of care taken to remove the causes which lead to the introduction of bacteria into it. Where dirty conditions prevail the milk may contain vast numbers of organisms; where careful methods are adopted the bacterial content may be greatly reduced. Freudenreich, Knopf, Orr, and others found an average of 50,000 to 80,000 bacteria per c.c. of milk collected in the cowshed soon after milking. In some few instances the number was as low as 5000, and

in others as high as 1,048,000, the variation being due to differences in the season at which the samples were taken, the cleanliness of the cows, the method of milking, and other factors.

With reasonable care it should be possible at most of the farms in this country to produce milk containing not more than about 10,000 to 20,000 bacteria per c.c. when it leaves the farm premises ; samples taken at the farm and found to contain more than 50,000 per c.c. should be considered as unnecessarily dirty and indicating negligence and disregard of precautions against contamination.

In addition to the pollution of milk which takes place at the farm, there is additional contamination during transit to the consumer. Bacteria are added during the railway journey, at the retailer's premises, on the milkman's rounds, and, finally, at the consumer's house.

Very many of the milk churns sent by rail are faulty in design, the lids allowing of easy access of dust and rain into the interior. The vans in which they travel are often dirty, and no special care is taken to unload or store the churns at properly cleaned depots, away from dust, foul odours, and other damaging influences.

When the churns are opened at the railway termini and small amounts transferred by means of imperfectly cleaned ladles or measures to the dairyman's cart, contamination occurs ; the same happens when the milk is poured over the rims of churns on which dust has collected or which have been handled by workmen whose personal cleanliness is not of the highest order.

Hewlett and Barton found from 20,000 to 8,390,000 bacteria per c.c. in milk samples taken at various railway

stations in London: Orr's figures for similar samples at several Yorkshire stations were 19,800 to 3,620,000, with an average of 232,600 per c.c.

At present milk is sold in all sorts of shops, some of them clean, but the majority unsatisfactory from the point of view of a clean milk supply. The vessels in which the milk is stored is, in many instances, open to the visits of flies and to the access of dust blown in from the street, the measures are not always clean, and, where the milk is stored in the living room of a house, there is considerable risk of infection with pathogenic organisms.

The estimation of the amount of contamination which takes place after the milk has left the farm, and due to the several causes enumerated, cannot be accurately made on account of the increase brought about by the growth and multiplication of organisms during transit to the consumer, this disturbing influence being especially active in summer, when the temperature is comparatively high.

According to Swithinbank and Newman's investigations, the milk retailed in London usually contains from 340,000 to nearly 5 millions per c.c.; in New York, Park found from 1 to 5 millions per c.c.

In the private houses of the consumer additions are made to the bacterial content, the amount varying with the place of storage, the cleanliness of the receptacles in which the milk is kept, and other factors.

Orr concluded from his investigations in Yorkshire that the farmer was responsible for about 40 per cent. of the organisms present in the consumer's milk, the rest, or 60 per cent., being contributed in approximately equal amounts by the dairyman, the consumer himself, and by pollution during the railway journey.

Ex. 125.—Determine the number of bacteria in a sample of milk.—Since the number of bacteria is usually very large in an ordinary sample of milk, it is necessary to dilute the latter in order to avoid too much crowding of the colonies on the plates which are inoculated by it.

For the purpose of dilution sterile water must be used.

It is usual to dilute the milk 10, 100, 500, or 1000 times.

Proceed as follows:—

From a burette run into a series of test-tubes 9 c.c. of water, and mark the level of the water in each tube with a file; add a few drops more, so that the water stands a little above the mark. Plug with cotton-wool, and sterilize by heating on three successive days for thirty minutes. (It will be found useful to have a series of small conical flasks marked at the 49 c.c. level, and a similar lot marked at the 99 c.c. level.)

Now take the sample of milk to be examined and shake it vigorously to obtain uniform distribution of its contents.

Remove with a sterile 1 c.c. pipette 1 c.c. of milk, and add it to the test-tube containing 9 c.c. of water. The milk is now diluted 10 times (1 in 10), and 1 c.c. of the mixture of milk and water contains $\frac{1}{10}$ of a c.c. of milk.

Gently shake the mixture for a short time, and then remove with another sterile pipette 1 c.c. from this first dilution and add it to another tube containing 9 c.c. of sterile water. The second dilution now obtained is 1 in 100, and 1 c.c. of it contains $\frac{1}{100}$ c.c. of the original milk. Shake and transfer 1 c.c. of this to 9 c.c. of sterile water, the dilution in the third tube is 1 in 1000, 1 c.c. containing $\frac{1}{1000}$ c.c. of the milk.

If a dilution of 500 times (1 in 500) is required, add 1 c.c. to 9 c.c. of water, and then 1 c.c. of this to a flask containing 49 c.c. of water.

Having prepared a suitable dilution, say 1 in 1000, add .5 c.c. of it to a tube of melted lactose gelatine from a sterilized graduated pipette, and, after gentle mixing, pour the inoculated medium into a Petri dish and allow it to solidify.

R

Inoculate three more plates in a similar manner, and incubate all at 18° to 20°; count the colonies appearing in seventy-two hours, and calculate the average number on each.

The counting can be facilitated by using a Pake's disc (Fig. 45) placed beneath the plates.

From the average number of colonies on a plate, the dilution

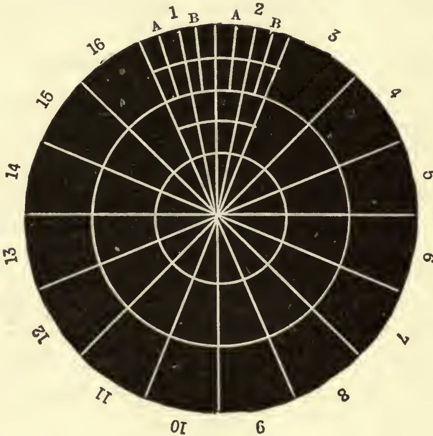


FIG. 45.—Pake's counting disc.

of the milk, and the amount of the latter used for inoculation, the number of the organisms in 1 c.c. of the original sample can be calculated thus:—

No. on plate × dilution × $\frac{1}{\text{amount used for inoculation}}$ = no. of organisms in the original sample.

E.g. If 420 colonies are found on a plate inoculated with .5 c.c. of the milk diluted 1000 times, the number present in 1 c.c. of the original sample is $420 \times 1000 \times \frac{1}{.5} = 840{,}000$.

Ex. 126.—Repeat the above but use a lactose agar medium instead of gelatine, and incubate at 37° C. for forty-eight hours.

Ex. 127.—Milk 50 c.c. of "foremilk," "midmilk," and "strippings" from the same quarter of the cow's udder into three sterile

flasks. Keep them cool, and after arrival at the laboratory determine as soon as possible the number of organisms in 1 c.c. of each by the method described in Ex. 125, using both lactose gelatine and agar media.

Ex. 128.—Take four sterile Petri dishes and pour into each the contents of a tube of lactose gelatine medium. Cover and allow the medium to solidify.

Prepare a similar number of lactose agar plates.

Take them to the farm; remove the covers and expose the surface of one of the gelatine and one of the agar plates for one minute:—

(1) In the open air.

(2) In the cowshed before the cattle are brought in for milking.

(3) Under or near the udder of the cow during milking.

(4) In the cowshed after it has been swept out.

After the stipulated time of exposure of the plates put on the covers, wrap them in paper and take them to the laboratory.

(*a*) Incubate the gelatine plates at 20° C.

(*b*) Incubate the agar plates at 35° C.

Count and compare the number of colonies appearing on the two media in 12, 24, 48, and 72 hours, noting the number which liquefy the gelatine.

Ex. 129.—Note the number of colonies on the plate which has been exposed for thirty seconds under or near the udder during milking, as in the preceding experiment. Calculate the area of the exposed surface of the plate by multiplying the square of half its diameter by 3.14. Find the area of the opening of the milking pail in the same way, and from this and the number of bacteria found to fall on the estimated surface of the plate in one minute calculate the number which fall into the pail during the whole time of milking.

Ex. 130.—Prepare Petri dishes of lactose gelatine and agar media.

Catch a fly in the cowshed, place it under the cover of the Petri dish and allow it to run over the surface of the gelatine or agar medium for one minute: then let it escape.

Incubate the plate at 20° C. and 37° C. respectively.

Count the number and note the kind of colonies which grow.

Try the experiment with flies caught in other places.

Ex. 131.—Place a cow's hair on the surface of a sterile lactose gelatine or agar plate.

Incubate for forty-eight hours, and note the growths which take place where the hair touches the medium.

2. **Influence of Temperature and Time upon the numbers of Bacteria in Milk.**—Although milk is an excellent medium for the nutrition of bacteria, their development in it is dependent upon suitable temperatures. Park found that when kept at freezing point (0° C.) for four to seven days the bacteria in it decreased slightly in number, and even at temperatures up to 6° C. growth was checked and little or no increase occurred. At higher temperatures, up to the optimum of 25° C. (77° F.), at which division goes on most rapidly, bacteria increase at an enormous rate, and milk in which only a small number are found initially may contain many millions in a few hours if kept in a warm place. This becomes obvious when it is remembered that a single bacterium which divides once every half hour can produce in twelve hours 16,777,216 offspring, provided that the temperature is suitable and the amount and nature of the medium will allow of such increase.

The following figures indicate the effect of time and temperature on the bacterial content of milk; the samples taken at the farm contained 22,000 bacteria per c.c.

Milk kept at	Number of Bacteria after			
	3 hrs.	6 hrs.	9 hrs.	24 hrs.
15° C.	27,000	66,000	110,000	3,850,000
25° C.	48,000	476,000	2,530,000	643,000,000
35° C.	751,000	2,000,000	8,360,000	112,500,000

Several observers have noted that in many cases the number of bacteria in milk decreases for a time after it is drawn from the cow. The length of time during which the decrease continues depends upon the kinds of organisms present and the temperature at which the milk is kept. When the temperature is low—say, about 10° C.—the decrease may continue for thirty-six hours or more; at 30° C. it lasts only for two or three hours. This peculiar fall in numbers and cessation of bacterial development, which occurs only during the first few hours in samples of fresh milk, has been attributed by some to the presence in the milk of a bactericidal substance which is soon used up or decomposed after it has left the udder. It may, however, be due to the death of many of the organisms which have come from the udder of the animal or from the surrounding air, owing to the altered conditions of existence to which they are exposed; those unaccustomed to live in milk die off or take time to become adapted to it.

After the bacterial content has reached its lowest point a steady rise sets in and proceeds more or less rapidly, according to the temperature, until a maximum is attained, about the time the milk becomes strongly acid and curdled. From this point the numbers begin to decrease again, and little by little the bacteria die off until

the milk, if kept for several weeks, is left in an almost sterile condition, or containing only moulds and similar fungi capable of tolerating the acidity of the medium.

Ex. 132.—Take about 250 to 300 c.c. of milk at the cowshed, and after thorough mixing pour 50 to 60 c.c. into four small flasks; label them Nos. 1, 2, 3, and 4 respectively.

Keep No.			
Keep No. 1	cool with ice.		for 6 hours.
„ 2	at a temperature of	10° to 15° C.	
„ 3	„ „	20° to 25° C.	
„ 4	„ „	35° C.	

After this, determine the number of bacteria per c.c. in each of the four samples by method described in Ex. 125.

Ex. 133.—Determine the numbers of bacteria in a sample of milk taken at the farm into a sterile flask as soon as possible after being drawn; then, at the following intervals, using both agar and gelatine media for the work:—

2, 4, 6, 12, 24 hours.
4, 8, 12, 24, 40, 80, 120 days.

The milk to be kept all the time in a tightly-corked flask at ordinary room temperature.

Note the appearance of mould colonies on the plates inoculated with the older milk.

3. **Methods of reducing the Numbers of Bacteria in Milk.**—To obtain a supply of milk with the least possible number of bacteria in it, necessitates constant attention to cleanliness on the part of all concerned in its production and distribution, and the maintenance of low temperatures so that the organisms unavoidably present in it cannot multiply to an objectionable extent.

It is not within the region of practical dairy farming to obtain a commercial supply of milk absolutely free

from bacteria, but it need not entail much trouble nor expense to provide wholesome milk which shall remain fresh and sweet under proper conditions of storage for twenty-four hours or so, within which time all ordinary supplies for domestic use ought to be consumed.

Although considerable increase in the bacterial content usually takes place after the milk has left the farmer's premises, this is probably more owing to the growth and multiplication of those introduced at the farm than to further additions made while the milk is on its way to the consumer. The investigations of Stevens and others upon market milk have shown that initial contamination has greater influence upon the bacterical content of milk delivered to the consumer than age and temperature. The primary infection at the farm and dairy should be checked, therefore, as far as possible, and this can only be accomplished by strict attention to the following suggestions.

In the first place, it is absolutely essential that only milk from healthy animals should be sent from the farm. All cows suffering from disease should be isolated from the milking herd, and no milk should be used for human consumption from tuberculous animals or those in any way affected with udder troubles.

A supply of good clean water is of great service on a farm. Not only is the health of the animals improved by it, but many of the worst milk troubles are avoided. Where cows are watered at dirty ponds in which they wade and stir up mud, various parts of their bodies become splashed with liquid containing bacteria, many of which sooner or later find their way into the milk and cause persistent trouble. Taints and bad keeping quality of the milk are sure to be encountered where

these conditions prevail, especially if the water used for washing the cans and other utensils is also impure.

The cows should be regularly groomed between the milking hours, all pieces of dung and loose hair being brushed off their udders, flanks, and haunches. Just before milking, the udders should be washed with tepid boiled water and wiped with a clean boiled cloth; the use of dirty cloths which have been allowed to lie about for sometime must be prevented.

The milkers should put on clean overalls and be compelled to wash their hands before milking. On no account should the filthy habit of "wet milking" be tolerated; the dipping of the hands into the fresh milk is a disgusting practice likely to lead to serious disease, and cannot be too strongly condemned.

The rejection of the first few jets from the teats, and the early removal of the milk from the cowshed to a clean cool place free from dust and away from the manure heap, greatly assist in keeping down the bacterical content.

Since dust and dirt are the chief carriers of bacteria every effort should be made to reduce these to a minimum. From the pail the milk should be passed through closely woven linen fabric or cotton wool strainers to take out all the larger particles of dung, straw, hair, and other materials which fall into it. The cowsheds should be white-washed at least twice a year, and the floors must be kept clean. The dung should be taken out as soon as possible and adequate means provided for the draining away of urine.

The brushing out of the stalls and the feeding of the cows with hay or dusty fodder should take place after milking—not just before—so as to avoid filling the air

with particles of dust which soon fall into and contaminate the milk

Too much attention cannot be given to the cleaning of the milk pails, churns, and other utensils, both by the farmer and the dairyman. The latter not unfrequently returns unwashed churns containing sour milk swarming with bacteria, to effectually get rid of which involves much trouble to the farmer.

All vessels should first be rinsed with cold water and then treated with live steam or boiling water. Rusty and badly indented cans, from the crevices of which it is difficult to clean out all the milk, should be discarded. Ample arrangements should be made for keeping clean cans in a cool clean part of the farm buildings, preferably in the dairy or cooling room.

The keeping qualities of the milk can be greatly improved by cooling it to 10° C. (50° F.) directly after it is drawn. If a cooler is used for this purpose it should be kept in a clean, dust-free place, and after use must be carefully cleaned: in any drops of milk left on it, bacteria increase enormously and may be washed off into the next milk which is passed over it.

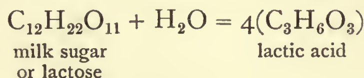
Churns or cans in which milk is sent by rail should be locked or sealed, and those patterns in which the lids allow some of the contents to splash out and run back again into the churn should be abandoned. The close-fitting, overlapping form of lid effectively prevents this from happening and at the same time does not allow rain to enter.

The provision of specially constructed refrigerator vans and cool, clean depots at the railway termini would do much to reduce contamination.

CHAPTER XVII.

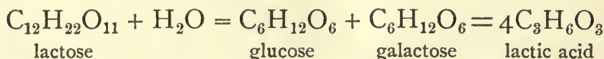
FERMENTATIONS IN MILK.

1. **Lactic Fermentation.**—When milk is first drawn from the cow it possesses a sweetish taste, although when tested by chemical means it is found to be slightly acid. If it is allowed to stand in a vessel for a day or two during warm summer weather, it becomes much more sour or acid and at the same time curdles. The curdling is due to the precipitation of the casein of the milk by the action of an acid which chemical examination has shown to be produced from the milk sugar. The acid is known as lactic acid (or oxypropionic acid, $C_2H_4(OH)COOH$), its production from the sugar being generally indicated by the following equation :—



$$\underset{\text{milk sugar or lactose}}{C_{12}H_{22}O_{11}} + H_2O = \underset{\text{lactic acid}}{4(C_3H_6O_3)}$$

It is probable that the milk sugar is first hydrolysed by an enzyme, *lactase*, the hexose sugars produced being subsequently acted on with the formation of lactic acid, thus :—



$$\underset{\text{lactose}}{C_{12}H_{22}O_{11}} + H_2O = \underset{\text{glucose}}{C_6H_{12}O_6} + \underset{\text{galactose}}{C_6H_{12}O_6} = \underset{\text{lactic acid}}{4C_3H_6O_3}$$

The change is brought about by the activity of bacteria which gain access to the milk after it is drawn from the udder of the animal. Theoretically the loss of

sugar in the fermentation process should be replaced by a definite amount of lactic acid, but the amount of acid produced is always less than would be expected from the above equation. Some of the sugar is used by the bacteria for nutrition and respiration with the formation of several compounds, such as acetic, formic, and succinic acids, and the liberation of the gases, carbon dioxide, hydrogen, nitrogen, and occasionally methane. The quantity of lactic acid used, and the amounts and nature of the by-products obtained, is dependent upon the species of organisms present in the milk and on the temperature and access of air. Under the most favourable conditions the yield of lactic acid is not more than about 89 per. cent. of what would be expected from the sugar which is lost, and is usually much less than this. At a temperature of 10° C. (50° F.) the lactic organisms grow very slowly and the amount of acid produced by them is very small. The best temperature for their physiological activity lies between 20° C. (70° F.) and 32° C. (90° F.). They are very sensitive to the action of free acids, .15 per cent. of hydrochloric or .03 per cent. of sulphuric acids completely check their work. Moreover, the fermentation of the sugar in milk generally ceases when the lactic acid present is over .8 per cent. before all the sugar is used up. The process can be made to advance further by neutralizing the acid with calcium carbonate as fast as it is produced.

Ex. 134.—Into one flask pour 500 c.c. of fresh milk and to another similar flask add the same quantity of milk and boil it for one minute: cool.

Leave both in a warm place and test the acidity of samples taken from each flask every twelve or twenty-four hours until the milk is curdled; shake it well before each test.

To do this, measure out with a pipette 25 or 50 c.c. into a porcelain dish. Add to it five drops of the following solution of phenolphthalein :—

Phenolphthalein	5 gr.
Alcohol, 90 per cent.	150 c.c.

(This solution turns pink when an alkali is added to it and remains colourless when acid : try it.)

Now run into the milk a decinormal solution of caustic soda from a graduated burette, carefully noting the point at which the caustic soda solution stands in the burette before beginning the experiment.

At first when the caustic soda solution touches the milk a pink coloration is produced, which disappears on stirring with a glass rod.

Run in more, drop by drop, until the pink colour is permanent, stirring vigorously after each addition of the soda ; when the pink colour remains for ten to twenty seconds after stirring, all the acid has been neutralized.

Read off on the scale of the burette how many c.c. of the soda solution has been used.

Calculate what percentage of acid was present in the milk, assuming that each c.c. of the soda solution corresponds to .009 gr. lactic acid.

Draw up a table showing the amount of acid in each sample of milk from day to day.

Lactic acid exists in three forms, all having the same chemical composition, yet differing from each other in molecular constitution and physical properties. A solution of one of these, known as *dextro-lactic acid*, has the power of affecting a beam of polarized light when passed through it, the plane of the light being rotated towards the right. Another form rotates the light equally to the left, when passed through solutions of the same strength ; it is termed *laevo-lactic acid.* When

mixed in equal proportions the resulting solution has no action on polarized light, one neutralizing the effect of the other. The acid in such a solution is spoken of as *inactive lactic acid.* Many kinds of the lactic bacteria produce the inactive acid in milk and solutions containing sugar and other carbohydrates. Formerly this substance was spoken of as *fermentative lactic acid.* Some organisms, however, are now known which produce dextro-lactic acid, others the laevo-lactic variety. Perhaps the majority produce the former, although the power of a certain kind of bacterium of producing a definite kind of lactic acid is dependent on the carbohydrate fermented as well as on the character of the medium and other factors. The acid produced under similar conditions may be used as a diagnostic character to assist in the discrimination of varieties or races of nearly allied kinds.

Over a hundred so-called species or kinds of bacteria have been described which have the power of producing lactic acid from various sugars and other carbohydrates. Some of them have not been found in milk, nor are they ever likely to occur in it. A very large number, however, have been obtained from milk and dairy products; and bacteriologists, who have been concerned with their investigation, have often bestowed on them different specific names in such a way as to create the impression that they were distinct and unrelated. There is little doubt, however, that many of them are closely related but slightly modified forms of the same organism, and that the number of fundamentally different species is comparatively few. Indeed, among organisms whose form, physiological power, and other diagnostic characters are so readily modified by their

environment, the term species as used when dealing with the higher plants and animals cannot be applied with any degree of precision. Well-marked, permanent, or constant differences which the term implies do not exist among the lactic bacteria. In the present state of knowledge it is, therefore, perhaps best to place those which have been described in groups according to the presence or absence of certain characters which are subject to comparatively little variation, the name of each group being that of one of the most distinct forms represented in it.

General Characters of lactic bacteria.—Most of the lactic bateria met with in milk and its products are cocci or short-rods, and are non-motile. They are without the power of spore formation, and consequently liquids containing only these forms are easily rendered sterile by heating to boiling point. Some forms of the lactic bacteria grow well enough on gelatine, while others exhibit feeble growth upon this or any other solid medium. Their colonies are generally either white or yellowish-white, and do not liquefy gelatine. They produce lactic acid in milk, especially at elevated temperatures, and usually curdle it sooner or later. Some grow well in the presence of oxygen, others, perhaps the majority of kinds, are facultative anaerobes.

The following groups suggested by Löhnis may be recognized, viz.:—

Group i. *Streptococcus lacticus*, Kruse.
„ ii. *Bacterium acidi lactici*, Hüppe.
„ iii. *Bacterium casei*, Freudenreich.
„ iv. *Micrococcus lactici acidi*, Marpmann.

The typical bacteria of groups i. and ii. are the most

frequent, being found in almost all samples of cows' milk.

(*a*) **Group i.**—The typical bacterium of this group, is that which is known as **Str. lacticus**, Kruse, and is the commonest of all forms occurring in milk. To it most of the souring in the dairy is due. It appears to be the same organism as :—

Bacterium lactis, described by Lister.
" *Guntheri*, L. & N.
" *lactis acidi*, Leichmann.
" *lactis acidi*, 1 and 2 of Conn.
Lactococcus lactis, Beijk.

The cells are usually slightly oval cocci, .6 to 1 μ long, and .5 μ broad, which before the process of division may lengthen slightly, so that it is difficult to decide whether they should be described as short bacteria or cocci. They are mostly in pairs or short chains of four or five together, hence the term *streptococcus* applied to them. This species is somewhat anaerobic and forms very minute colonies below the surface of gelatine plate cultures. Under any circumstances the growth is very slight on solid media, and the colonies are mostly round, $\frac{1}{4}$ to $\frac{1}{2}$ mm. in diameter, about the size of a small pin's head, but may be oval or irregular in outline sometimes. The organism stains with Gram's stain, and does not produce gas when inoculated into fermentation tubes containing broth and sugar.

In milk it grows most rapidly at a temperature of about 30° to 35° C., and the forms generally met with produce acid and curdling in six or eight hours, the curd being dense and uniform, without gas bubbles and no separation of whey. Some varieties are slow in action at this

temperature. Those isolated from fresh milk often give rise to lactic acid, feebly at first, but their power in this respect increases very considerably when they are cultivated in milk for some time. The soured milk has a pure, clean taste, occasionally with a fine distinct aroma, the acid being chiefly right handed lactic acid with little or no acetic or volatile acid by-products. *Str. lacticus*, or forms of it, are the most useful of all lactic bacteria for dairy purposes, and the majority of the "starters" utilized in manufacture of butter and cheese are more or less pure cultures of this type.

FIG. 46.—*Streptococcus lacticus*, Kr. (× 800).

Streptococcus lacticus, Kruse (Fig. 46).—The following morphological and biological features are characteristic of the type:—

Size and form.—A streptococcus or short bacterium .5 μ to 1 μ in length. Stains by Gram's method.

Gelatine.—The colonies are white and yellowish sometimes, very minute, and grow only below the surface.

Stab growth is slight, usually beaded or granular; no surface growth.

Agar.—Very small, little or none on surface.

Potato.—Little or no growth.

Bouillon.—Very slight growth, with the faintest turbidity and sediment.

Milk.—Acid without gas: curd dense, smooth, and firm, without separation of whey. No peptonization of the casein.

Included in Group i., and very nearly allied to *Streptococcus lacticus*, are a number of other bacteria, differing in some particular characters from the above description. *Streptococcus Kefir.*, Mig., from Kefir, and some others

produce acid without coagulation of milk. *Str. hollandicus*, Scholl, found in the Dutch "lange Wei" (see p. 279), and *Micrococcus mucilaginosus*, Mig., form slimy material. There are others which set free slight amounts of gas during fermentation, and are negative to Gram's stain. To these may be added *Str. mastitidis*, Guill., a form causing epidemic inflammation of the udder of cows, the affected animals yielding small quantities of yellowish milk: pure cultures coagulate milk and set free small amounts of gas. Related to this group is the pathogenic, *Str. pyogenes*, Rosenbach, which sets up several inflammatory diseases in animals.

(*b*) **Group ii.**—The typical organism of this group is that described by Hüppe, under the name of **Bacterium acidi lactici.** It is widely distributed in milk but rarely in any abundance, its development being probably checked by organisms of the *Str. lacticus* type. The chief character distinguishing it from the bacteria of group i. is its power of fermenting milk sugar and other carbohydrates, with the production of considerable amounts of carbon dioxide gas, often mixed with a little hydrogen. The organism is always rod-shaped, but varies much in length. Perhaps the average dimensions are 1 to $1\frac{1}{2}\mu$ long, and $\frac{3}{4}$ to 1μ broad, the cells may, however, reach a length of 10 or 15 μ and $1\frac{1}{2}$ or 2 μ broad, and in certain media undergo peculiar changes in form. They are usually united in pairs, but some forms may appear as chains of stumpy cocci.

Bact. acidi lactici grows best aerobically and forms rounded white, slimy colonies on the surface of gelatine plates. They are often 5 or 10 mm. in diameter, much larger and very different from the colonies of *Str. lacticus*. The bacteria stain faintly with Gram's stain. When

introduced into milk, souring takes place rather slowly. In a day or two at a temperature of 30° to 40° C., coagulation occurs, and the curd which is precipitated contains a few small gas bubbles, the serum which separates being clear. The taste and odour developed are generally disagreeable. Lactic acid is formed and often with it occurs a fair amount of acetic acid as well as traces of formic and succinic acids and alcohol. Many troubles of the dairyman in butter-making and cheese-making can be traced to organisms belonging to this group.

The following are the chief characters of typical **Bact. acidi accici**, Hueppe.

Size and Form.—Rods 1 to $1\frac{1}{2}$ μ long and $\frac{3}{4}$ to 1 μ broad in pairs and chains.

Gelatine.—Surface colonies round or lobed, white and moist and slimy, very similar to those of *B. coli.* Deep colonies, small, usually spindle-shaped.

Agar.—Irregular, greyish, shining; deep colonies, small, round or spindle-shaped.

Potato.—Abundant growth, greyish at first, then yellow or brown.

Bouillon.—Turbid with abundant sediment.

Milk.—Acid produced and curd precipitated with a few small bubbles in it.

Sugar Bouillon.—It ferments glucose and lactose with acid and gas production, but not saccharose.

Indole is produced by it in peptone solution (Ex. 63), but it does not give the Voges and Proskauer reaction (Ex. 65).

A form which appears to be almost as common as the type already described and very closely allied to it, is known as **Bacterium lactis aerogenes**, Esch. It was first isolated from the milk-stools or fæcal matter of

infants, but is often met with in milk, although rarely in any abundance. The colonies on gelatine plates are round, shining, white like porcelain, with smooth, circular margins, and more or less hemispherical, standing up from the surface of the plate. The bacteria from typical cultures do not stain by Gram's method (see p. 8). Some forms in this group very closely resemble *Bacterium coli* (p. 106), and are difficult to distinguish from the latter with certainty. Eckles' Bacillus No. 8, which gives a pleasant, ripe cheese aroma in milk, and is a useful "starter" for the butter-maker, appears to be a form near *Bact. lactis aerogenes*. The *Bacilli Guillebeau*, *a* and *b* (Freudenreich), obtained from milk of cows suffering from inflammation of udder, and giving rise to "gassy" or "blown" cheeses, are closely related to *Bact. l. aerogenes*. In the same group there are included many other bacteria, isolated from milk by various workers, which differ slightly from the typical form described above. *Bact. limbatum*, Marpmann, coagulates milk but produces no gas. *Bs. lactis innocuus*, Wilde, and Bs. No. 41, Conn, give rise to a little acid only, but do not coagulate milk or develop gas in it. The latter variety gives rise to a pleasant aroma in unsterilized milk. Several varieties which have been isolated from cheese should be included here. Very closely resembling *Bact. acidi lactici*, Hüppe, and therefore to be included in the group is *Bact. pneumoniæ* of Friedländer, the cause of pneumonia in man. It does not coagulate milk, but forms gas. When taken from the body or from cultures in milk the organisms exhibit well-developed gelatinous capsules. The colonies on gelatine are round, moist, and white, very similar to those of *Bact. lactis aerogenes*. To the same group may be added *Bact. lactis viscosum*,

Adametz, a variety which renders milk slimy and causes trouble in dairies sometimes.

(*c*) **Group iii.**—In this group the typical form may be taken as **Bacterium casei**, Freudenreich. The organism is a thin, elongated rod, 2 to 3 μ long and .5 to .7 μ broad. Most of the forms in this group grow feebly and are usually more or less anaerobic. Their colonies are small, about the size of a pin's head, white or yellowish-white, and variable in shape. The typical *Bact. casei* produces inactive lactic acid in milk and coagulates the latter in three days at a temperature of 30° C. with gas formation. Allied to it are *Bact. casei*, Leichmann, and *Bact. lactis acidi*, Marpmann, which do not produce gas in milk. *Bs. caucasicus*, Beijk. (p. 315), one of the active fermentation agents in Kefir grains, and *Bs. bulgaricus*, Heupel. (p. 319), should probably be included in this group. Other allied forms are known which neither precipitate casein nor form gas.

(*d*) **Group iv.**—The type species or form of this group is **Micrococcus lactis acidi**, Marpmann. It is a small coccus .6 to .8 μ in diameter, occurring singly or in pairs and small heaps, but never in chains. In milk it grows slowly, rendering it acid at 20° C., but not coagulating it below 30° C. The colonies on gelatine are small and round, yellowish-white in colour, growing chiefly on the surface of the plate. No gas is formed. *Micrococcus pyogenes*, Rosenbach, and its varieties, which are associated with the formation of pus in various inflammatory and suppurative diseases, belong to this group. They coagulate milk and tend to produce enzymes, which dissolve casein and liquefy gelatine. In some varieties the colonies are white, in others different shades of yellow or orange. The organisms producing

bitterness in cheese, *Micrococcus casei amari*, Freudrch., and Conn's *Micrococcus* of bitter milk, belong to this group also.

Ex. 135.—Inoculate tubes of sterile milk with pure cultures of :—

(*a*) *Str. lacticus*, Kr.
(*b*) *Bs. acidi lactici*, Hueppe.
(*c*) *Bact. lactis aerogenes*, Esch.
(*d*) *Bact. coli*, Esch.
(*e*) *Bs. bulgaricus*, Hpl.

Note the kind of curd thrown down by each, and the presence or absence of gas bubbles in it.

Ex. 136.—Make cover-films from pure cultures of the organisms mentioned in the previous experiment, and stain them with :—

(*a*) weak gentian violet,
(*b*) Lœffler's methylene blue,
(*c*) carbol fuchsin,
(*d*) Gram's stain.

Examine with the highest power available and make drawings of the organisms.

Ex. 137.—Mix a few drops of sour milk in a test-tube with sterile water.

Inoculate tubes of melted litmus gelatine and litmus agar with a loopful of the diluted milk, and pour into separate Petri dishes.

(*a*) Incubate the gelatine plates at 20° C., the agar plates at 35° C., and note the number, form, and acidity of the colonies obtained.

Do any of them liquefy the gelatine?

(*b*) Inoculate tubes of litmus milk with small portions of the different colonies observed.

Incubate at 20° C. and make notes on the following points during seven to fourteen days :—

1. The presence or absence of curd.

2. The texture of the curd thrown down.
3. The separation of whey.
4. The presence or absence of gas bubbles.
5. The odour of the fermented milk.

Do any of the organisms peptonize or soften and digest the curd?

(*c*) With portions of the different colonies isolated above—

Inoculate an agar slant.
,, a potato slice.
,, a gelatine stab.
,, a tube of glucose broth in a Durham's tube.
,, ,, lactose ,, ,, ,,
,, ,, saccharose ,, ,, ,,

Test their nitrate reducing power (Exs. 74 and 82).
,, indole production (Ex. 63).

Try Voges and Proskauer's reaction with each (Ex. 65).

Record the characters of each in accordance with the scheme given on pp. 60-61.

2. **Slimy or Ropy Fermentations of Milk.**—Many liquids, such as grape juice, beer, cider, and various plant infusions which contain sugar, sometimes become ropy or slimy, so much so that they may be drawn into long gelatinous strings. A similar production of a slimy substance is not unfrequent in milk and cream, and is brought about by the action of bacteria, most of which appear to be introduced into the dairy in the water supply or in dust from hay and other food materials. The chemical nature of the ropy material has not been exactly determined. It appears to arise, however, either :—

(1) from the milk sugar which is changed into a gum-like carbohydrate ;
(2) from the production of a slimy protein substance

from the casein, which substance may be set free in the serum or watery part in the milk, or

(3) from the presence of swollen gelatinous capsules round the organisms.

Possibly the slimy character of the medium is due in certain cases to the enormous number of bacteria developed in it.

Many species or varieties of bacteria are capable of producing varying degrees of ropiness in milk and cream. In some instances the milk or the milk serum becomes ropy in a few hours, in which case the cream is of normal consistency: in most samples, however, generally the change occurs only after a considerable period, and is therefore a commoner phenomenon in cream, and especially when the latter is raised in pans. Ropiness is less frequent in "separator" cream.

The slime-producing organisms which have been most carefully investigated and described are referred to below.

(*a*) **Streptococcus hollandicus**, Scholl, known as the "Ropy Whey" or "Lange Wei" organism, is a coccus which occurs in pairs and longer chains. It is allied to the ordinary *Str. lacticus*, and, like it, produces lactic acid, but has the additional power of rendering milk stringy and capable of being drawn out into long threads in from twelve to fifteen hours at a temperature of 22° C. The slime appears to be a product of the albumin of the milk. Ropy Whey is used as a "starter" in Holland in the manufacture of Edam cheese. The colonies on milk-peptone gelatine are very small: they do not liquefy the medium.

(*b*) **Micrococcus Freudenreichii**, Guillebeau, is a very large aerobic coccus, often nearly 2 μ in diameter, which produces stringiness in milk in a few hours at a temperature of 20° C. or less. Its colonies on gelatine are very small, round, and white,

the medium soon becoming liquefied: on potato they are pale yellow.

(*c*) **Bacterium lactis viscosum**, Adametz, is a non-motile, coccus-like bacterium, with a well-developed capsule, about 1 μ in diameter, and producing slimy whitish colonies on gelatine and agar somewhat like those of *Bact. lactis aerogenes.* It sometimes occurs in threads 2 to 5 μ long. When introduced into milk it renders it slightly alkaline and viscous in a day or two, but the stringy condition does not develop until a much longer time has elapsed. The milk sugar is not much altered, the slimy substance being apparently produced at the expense of the casein of the milk. Cream inoculated with it becomes slimy if kept some time, and butter made from such cream is soft and does not keep well. The bacterium is generally introduced to the dairy in the water supply, and is one of the commonest causes of ropy milk.

Resembling this species is *Bs. lactis pituitosi*, Löffler, a thick bacillus which breaks into coccus-like segments and renders milk slightly acid.

(*d*) **Bacterium Guillebeau**, *c*, Freudenreich, is a type of bacterium isolated from milk yielded by cows suffering from inflammation of the udder. Milk is rendered slimy by it. It produces colonies on gelatine like those of *Bact. lactis aerogenes.*

(*e*) **Bacterium Hessii**, Guillebeau, a motile form 3 to 5 μ long, 1 to 2 μ thick, nearly related to the hay bacillus (*Bs. subtilis*), makes milk ropy, but the ropiness gradually disappears when acidity develops.

(*f*) **Bs. viscosus**, I. van Laer, a bacillus 1.6 to 2.4 μ long, .8 μ thick, isolated from beer, in which it produces slimy material, can produce ropiness in milk. It forms small white colonies on gelatine plates, which at first are round, becoming slimy with irregular margins later.

(*g*) **Streptococcus hornensis**, Boek., a coccus isolated from slimy milk in Holland, and found in separator slime, water, and on flowers. It produces small white colonies on gelatine. Introduced into milk containing cane sugar at 22° to 30° C.,

the liquid is rendered very ropy, the slimy product being the gummy carbohydrate dextran.

(*h*) An organism very closely resembling *Str. lacticus* was found by Burri to be the cause of slimy milk and whey in a Swiss dairy. He traced it to the milk ducts in the udder of some of the cows.

3. **Development of Colour in Milk.**—Many bacteria possess the power of manufacturing colouring matter, which may become distributed in the media in which they are growing, colouring them red, pink, yellow, blue, or some other tint. They may occur in milk, although colour production through the agency of bacteria is rarely met with in this liquid, and then only when it is kept a considerable time. The following varieties of coloured milk are best known :—

(i) **Red Milk.**—It is not very uncommon to meet with milk having a reddish tinge or which deposits a reddish sediment after standing for a time in a can or other vessel. The colour is due to the presence of blood derived usually from the rupture of a small blood-vessel in the udder of the animal. In such cases the red blood corpuscles are readily detected by the microscope (see p. 302). In some instances, however, the red colour is not visible in the milk freshly drawn, but develops slowly and increases slightly from day to day. Such examples are due to the growth of certain species of bacteria, of which the following have been isolated and studied :—

(*a*) **Bacterium erythrogenes**, L. & N. (*Bacillus lactis erythrogenes*, Grotenfelt). The organism is non-motile and rod-shaped, .8 to 2 μ long and .5 to .8 μ thick. When grown in milk its coagulation occurs slowly, the serum separates from the

casein and the cream becomes blood-red in colour and slightly alkaline. The colonies liquefy gelatine and produce a red coloration upon it, and upon agar when grown in the dark. In the light the colonies are yellowish.

(*b*) **Micrococcus roseus**, L. & N., is a very common bacterium found in the air. It grows readily in milk, forming a red sediment in the latter without otherwise effecting much change. Nearly allied to this is *Sarcina rosea*, Schröter, which has been found to give a red coloration to milk, with slow precipitation and solution of casein.

(*c*) **Bacillus prodigiosus**, Flügge (L. & N.) (*Micrococcus prodigiosus*, Cohn), is also a common air and water organism which appears as a large coccus 1 μ in diameter on solid media and as rods in liquid media. It is sometimes motile, and when grown on potato and agar produces rose-red to reddish-purple colonies. It liquefies gelatine and the colonies are often greyish-white instead of reddish in colour. On the surface of milk or cream it may produce its characteristic blood-red pigment, but in the liquid it precipitates and dissolves the casein with the production of a faint yellow colour only.

(ii) **Yellow Milk.**—A number of bacteria have been found to give rise to a yellow or orange coloration in milk, especially if the latter is kept for a long time. The species, which has been most carefully investigated, is

Bacterium synxanthum, Ehr, a short, thin rod-like organism obtained from boiled milk, in which it gives rise to a bright yellow colour; the casein is dissolved, the solution becoming alkaline. In acid milk the colour is not developed.

(iii) **Blue Milk.**—The development of a sky-blue colour in milk has occurred from time to time in certain dairies, especially on the Continent, and so far back as 1838 investigations were made to determine its cause. That it was due to the work of micro-organisms was demon-

strated by Fuchs in 1841. The colour is due to the activity of a bacterium named

Bacillus lactis cyanogenes, by Hüppe (*Bact. syncyaneum*, L & N.), a motile aerobic rod-shaped form 1 to 3 μ long and .5 μ thick. After introduction into fresh milk it induces a blue colour in from one to three days, or earlier if the temperature is maintained at about 20° C. The colour appears first at the surface of the milk in small spots or patches, and spreads slowly into the deeper parts. The production of a rich deep blue tint is dependent upon the simultaneous production of a certain amount of acid by the lactic organisms, although the acid is removed in some way almost as fast as it is formed, since the coloured milk has an alkaline reaction. On keeping, the colour changes to a dirty grey or brown. The colonies on gelatine are usually yellowish- or greyish-white at first, but later become brownish purple. It is easily checked by the presence of other bacteria, and is destroyed at a temperature of 60° or 70° C. A greyish tinge only is developed by the blue milk bacillus in sterilized milk or in fresh milk which has not become acid.

Ex. 138.—Prepare stained slides of the following organisms :—

Bact. lactis viscosus, Adamtz.
Str. hollandicus, Sch.
Micrococcus roseus, L. & N.
Bs. prodigiosus, Flügge.
Bs. lactis cyanogenes, Flügge.

a. Examine with high power, and make drawings of each.
b. Try their action upon (1) fresh milk, and (2) milk to which sugar has been added.
c. Grow them upon gelatine and agar media, and note the form and colour of the colonies.

4. **Bitter Milk.**—The bitter flavour of milk sometimes appears to be brought about through the consumption by the cows of leaves of turnips and other cruciferous plants, lupins, and vetches, as well as certain

weeds, such as chicory, tansy, and wild chamomile. Cows with udder disease also yield bitter milk occasionally.

In all these instances the bitterness is recognisable in the milk as it is drawn from the animal, and it does not increase with age of the milk.

There are, however, many cases recorded of serious bitterness of milk which have originated through the activity of bacteria.

In boiled milk the bitter flavour not infrequently arises from the spore-bearing organisms, such as *Bs. subtilis* and its allies, as well as some of the butyric acid organisms: in unboiled samples these bacteria are generally kept in check by the lactic acid organisms.

In fresh milk bitterness may arise; the trouble in many cases has been traced to the contamination with various kinds of bacteria. Varieties of *Bact. coli* and *Bact. lactis aerogenes* have been found capable of inducing the objectionable change in milk, the peculiar taste being brought about in most cases by decomposition of the protein of the milk. Freudenreich isolated from bitter cheese a small coccus, which he named *Micrococcus casei amari*. It liquefies gelatine, renders milk acid, and coagulates the casein, producing at the same time a bitter taste.

The same writer described a motile bacillus obtained from bitter cream which liquefies gelatine and gives rise to an intensely bitter flavour in milk, and coagulates it without rendering it acid.

Eckles has also described a small oval coccus capable of giving rise to bitterness in milk, and rendering cheese made from it very bitter (see p. 377).

Harrison traced the bitterness of milk in a cheese factory to contamination with a species of *Torula* or non-sporing yeast (see p. 377).

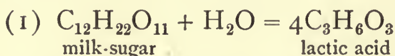
5. **Butyric Acid Fermentations.**—When a mixture of milk, old cheese, sugar, and chalk or calcium carbonate is kept warm fermentation soon begins; a great evolution of gas occurs, and butyric acid is formed, which, when neutralized by the chalk, yields calcium butyrate. In the process lactic acid or calcium lactate is first produced, and this is fermented subsequently with the formation of calcium butyrate.

The nature and cause of this and similar reactions where carbohydrates are broken down with the production of butyric acid were studied by Pasteur, Van Tieghem, and others. They showed that the fermentations were brought about by the action of a group of anaerobic bacteria which are widely distributed in water, milk, cheese, soil, dung, and other materials.

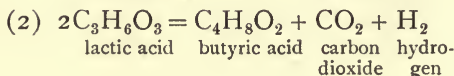
A large number of butyric acid bacteria have been described since Pasteur's time as if they were distinct, but the recent investigations of Beijerinck, Schattenfroh, and Grassberger have shown that most of those described by earlier writers are varietal forms of a few distinct species only.

In the fermentation of calcium lactate this substance is changed into calcium butyrate, but at the same time certain bye-products are formed in variable amounts; the gases carbon dioxide and hydrogen are given off, and small quantities of ethyl and butyl alcohol are frequently formed along with propionic and other acids.

The two stages in the change of milk-sugar into butyric acid may be expressed thus:—



$$(1)\quad \underset{\text{milk-sugar}}{C_{12}H_{22}O_{11}} + H_2O = \underset{\text{lactic acid}}{4C_3H_6O_3}$$



$$(2)\quad \underset{\text{lactic acid}}{2C_3H_6O_3} = \underset{\text{butyric acid}}{C_4H_8O_2} + \underset{\text{carbon dioxide}}{CO_2} + \underset{\text{hydrogen}}{H_2}$$

In addition to the indirect butyric acid fermentation of milk sugar, direct fermentation of this and other sugars without the preliminary change into lactic acid can be carried out by several organisms; other bacteria are known which produce butyric acid from starch, citric, malic, and other acids, and from glycerine.

It is usual to limit the term butyric acid fermentation more particularly to the production of this acid from carbohydrates and glycerine. Most of the organisms possessing this power are anaerobic bacilli, which in a vegetative state store up *granulose*, a kind of carbohydrate giving a blue or violet colour with iodine, like that given by starch. They form highly resistant spores, which may be heated to the temperature of boiling water without being destroyed. These often appear in the middle of the bacterial cell, and when fully developed bulge out the walls of the latter into the form of a spindle; a bacterial cell of this shape is usually termed a *Clostridium*.

Besides the above typical butyric acid fermentations in which a carbohydrate is broken down, with the formation of butyric acid as a chief product, many fermentations are known in which butyric acid in larger or smaller amounts arises in the decomposition of proteins, the organisms concerned belonging mainly to the putrefactive class, most of them being closely allied to *Bs. vulgatus*.

Butyric Acid Bacteria.—1. Great uncertainty exists in regard to the relationship of the butyric acid organisms described by many different writers; Pasteur's *vibrion butyrique*, and Van Tieghem's *Bacillus Amylobacter*, were considered by Prazmowski the same as his *Clostridium butyricum*. It is not feasible here to give an account of the various forms which have been described. Most of

them, although differing in name, are no doubt similar organisms; it will suffice to briefly describe the two main species isolated and studied by Schattenfroh and Grassberger, since these two species apparently include most of the anaerobic forms which attack carbohydrates.

a. Species I., described as "**motile non-liquefying Granulobacillus saccharobutyricus**," is apparently the same organism as *B. saccharobutyricus* of Von Klecki, *Granulobacter saccharobutyricum* of Beijerinck, *B. amylozyma* of Perdrix, and is closely allied to the pathogenic organisms *B. Chauvoei*, which causes blackleg in cattle, and *B. enteritidis sporogenes*.

It is common in water, soil, meals of various cereals, and comparatively rare in milk.

The bacteria are rod-shaped, with peritrichous flagella; they grow best under strict anaerobic conditions at 35° to 37° C. Clostridia are formed containing oval spores, 1.8 to 2.3 μ long and 1.3 to 1.7 μ broad.

The organism converts milk-sugar almost completely into butyric acid, carbon dioxide, and hydrogen, little or no lactic acid being formed; from grape-sugar, however, it forms both acids.

Proteins are not affected by it.

Gelatine is not liquefied. In grape-sugar gelatine media stab-cultures may exhibit three types of growth, viz., (*a*) A beaded track with the organisms in the *Clostridium* form; (*b*) a root-like track, with fine outgrowths; (*c*) a turbid track, showing gas bubbles, the organisms motile and without granulose.

Grape sugar agar.—In stab-culture much gas is produced, and a strong smell of butyric acid.

Potato.—Extensive growth, white and frothy, with strong odour of butyric acid. The bacteria contain much granulose.

Milk.—In milk butyric acid is produced, and casein, full of gas bubbles, is precipitated, but not peptonized.

Bouillon.—In ordinary peptone broth little development occurs, but it grows rapidly in glucose peptone solution.

b. The second species, described as the "**non-motile Granulobacillus saccharobutyricus,**" is a common organism in milk, dung of farm animals, and human fæces; it is also abundant in water, soil, and the meal of cereals.

Two varieties exist, one a long cylindrical bacillus with rounded ends, often in chains three to six together; the other a smaller and shorter organism, rarely united in chains.

Both are strictly anaerobic.

In the fermentation of milk-sugar, and carbohydrates by these organisms, lactic as well as butyric acid is formed, often in larger quantity than the latter compound.

Granulose is present in the growing cells, but generally absent from those in which spores are formed: this substance is most abundantly produced when the organism is supplied with starch, little or none being made when sugar is supplied.

The spores withstand boiling for an hour and a half.

Gelatine media are liquefied.

Agar.—Surface colonies on slightly alkaline agar, containing 1 grain of starch or sugar per litre, are smooth and shining, irregular in outline in the case of the long variety, rounder and more watery in the short-celled form.

Milk is rendered acid: the casein is coagulated but not peptonized; it becomes full of gas bubbles.

Bouillon containing 2 per cent. of starch or grape-sugar is a good medium for the growth of this organism, but spores are not produced in it.

Ex. 139.—To obtain Clostridium-forms of *Granulobacillus saccharobutyricus*, the following receipt, suggested by Beijerinck, gives successful results :—

Put in a flask :—

Cane-sugar	10	gr.
Finely-ground fibrin	10	„
Precipitated calcium carbonate . .	6	„

Sodium phosphate	.1 gr.
Magnesium sulphate	.1 ,,
Sodium chloride	.1 ,,
Water	200 c.c.

Boil briskly for two or three minutes, and while boiling add 10 to 15 grams of fresh garden soil.

Plug loosely with cotton wool and transfer while hot to an incubator kept at 35° C.

The heating destroys moulds and non-sporing bacteria and drives out dissolved air: the spores of the butyric acid bacteria resist boiling, and, as the liquid cools, germinate and grow, the necessary anaerobic conditions being maintained to a certain extent by resistant aerobic bacteria introduced in the soil.

Examine the sediment at the bottom of the flask daily for a week as follows:—

(*a*) Tilt the flask gently and remove a small portion of the slimy deposit with a platinum loop.

Add it to a drop of a dilute solution of iodine in potassium iodide on a glass slide: put on a cover-slip and examine with a high power.

The butyric organisms containing *granulose* are stained a violet or violet-blue colour.

Look out for clostridial forms with spores in them; the latter appear as unstained, bright, oval or round bodies in the clostridial-cells.

(*b*) When spores are found mix a small amount of the slimy deposit with two or three drops of water in a watch-glass: then spread a drop or two of the mixture on two or three cover-slips Allow to dry: then stain spores by method given in Ex. 12.

Ex. 140.—Half fill a small yeast flask with the solution mentioned in the previous experiment.

Close the neck with a rubber cork through which passes a narrow bent glass tube arranged as in Fig. 47.

Now boil the solution for two to three minutes: and while boiling add 10 to 15 grams of fresh garden soil.

Connect with a short piece of rubber tube to a hydrogen generator and pass the gas through the solution for a few minutes: then clamp the rubber connecting tube and disconnect the hydrogen apparatus.

Dip the open end of the side tube of the flask into a small beaker or cylinder containing mercury and transfer to the incubator at 37° C.

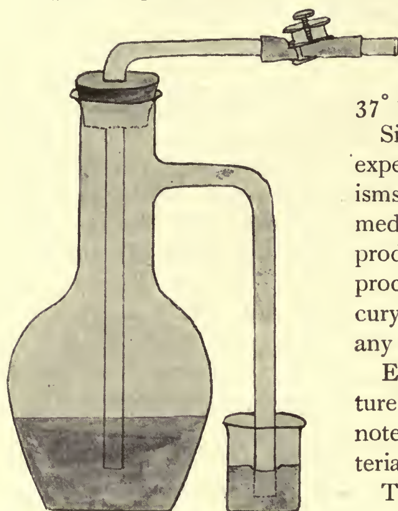


FIG. 47.—Yeast flask arranged for cultivation of anaerobic organisms.

Since the oxygen has been expelled the anaerobic organisms can now grow in the medium, and any gas which is produced in the fermentation process can escape by the mercury valve without setting up any great internal pressure.

Examine a drop of the culture after forty-eight hours and note the character of the bacteria present.

Test for granulose with iodine in potassium iodide.

If *Clostridia* with spores are present, stain the latter by method given in Ex. 12.

2. Although it is advisable perhaps to limit the term butyric acid fermentation to the production of this acid from carbohydrates, lactic acid, or glycerine, it must be noted that it is a frequent product in the decomposition of proteins by bacteria.

In the former process butyric acid is one of the chief substances obtained, while in the latter the amount is usually small and of secondary importance.

Many of the organisms which give butyric acid when breaking down proteins are aerobic.

(*a*) Belonging to this class is **Bacillus butyricus**, Hueppe, an aerobic bacterium occasionally present in milk, and said to be able to produce butyric acid from milk-sugar after this compound has been hydrolysed by other organisms.

It is a rod-shaped bacillus 8 to 10 μ long, 1 to 1.2 μ broad; stains by Gram's method and has large oval spores.

Gelatine.—The colonies are yellowish-white; liquefying stab-cultures soon show deep funnel-shaped liquefied portions.

Agar.—Yellowish-white slimy colonies, rounded and slightly elevated.

Potato.—A slimy yellowish-brown growth.

Milk.—Coagulated and rendered alkaline, casein peptonized; neither gas nor indole are produced, but a small amount of H_2S is formed.

(*b*) In this group should be included **Bacillus vulgatus** (p. 126) and probably other putrefactive aerobic soil bacteria, though the amount of butyric acid which they produce is small.

(*c*) Emmerling isolated an aerobic bacillus (**Bacillus boocopricus**) from cow dung which formed butyric acid from glycerine; gelatine was not liquefied by it.

CHAPTER XVIII.

FILTRATION; COOLING; PASTEURIZATION AND STERILIZATION OF MILK; PRESERVATIVES.

THE suggestions made in Chapter XVI., § 3, have reference chiefly to the production of a clean milk—that is, they aim at protecting it from becoming contaminated with bacteria. It is, however, important to deal with another side of the question, namely, the methods to be adopted in order to remove or destroy those organisms which unavoidably gain an entrance into it, or to check their development.

Three methods may be specially mentioned:

(i) The filtration of the milk;
(ii) the application of low or high temperatures,
(iii) the introduction of "preservatives" or soluble chemical substances which inhibit the multiplication of bacteria.

(i) **Filtration.**—Strainers consisting of various combinations of cotton or linen fabrics placed over fine wire gauze should always be used at the cowshed or dairy, to remove the particles of dung, hair, hay, and other refuse which fall into the milk. Milk may be filtered through layers of gravel, sand, and cloth, which remove a considerable proportion of the bacteria present, although they do not take out all of them. The difficulties connected with the cleaning and renewal of such filters, as

well as the comparative slowness of the operation, have prevented their general adoption. Germ-free milk for laboratory purposes can be obtained by filtration through porcelain filters, but much of the fat is removed in the process as well as the bacteria.

(ii) **The Effect of Low and High Temperatures.** (*a*) **Cooling.**—One of the most effective means of preventing milk from undergoing fermentative changes is to cool it and keep it at a low temperature. The sooner this can be done after the milk is drawn from the cow the better. Most of the bacteria which damage milk, from the consumer's point of view, grow very slowly at 10° C. (50° F.), and where it can be cooled immediately after milking, and maintained at this or a lower temperature, it will keep without any recognizable change for several days. Moreover, cooling does not appreciably alter the flavour of the product.

It must be noted that the bacteria are not killed by this treatment, nor can they be killed by the application of the lowest temperatures attainable: they are merely checked for the time, and will develop readily enough when the temperature is raised.

(*b*) **Sterilization.**—The sterilization of milk can be secured by heating it under pressure to a temperature of 110° C. or 115° C. (230° to 240° F.) for half an hour. Boiling it for twenty minutes destroys all the common forms, but the spores of certain species which are conveyed into milk by particles of hay and soil resist the treatment for some time, and may ultimately grow and set up objectionable chemical changes in it when the temperature is reduced. However, continuous boiling for an hour or two will destroy the spores of all bacteria, and render it sterile.

The sterilization of milk in this way has not become a popular process, partly because of the trouble and expense involved, but mainly on account of the alteration in flavour which results. The taste of boiled milk is very readily recognized, and is disliked by children as well as by adults. Nevertheless, in spite of the disadvantages and objections to the use of boiled milk, boiling for a short time is the simplest and safest means of getting rid of pathogenic organisms, which, under present conditions of production and distribution, too frequently find their way into the milk supplied to the householder.

Ex. 141.—Pour 250 c.c. of fresh evening's milk into a sterile flask, and allow it to stand in a cool pantry until next morning.

(*a*) Dilute a small amount of it to 1 in 1000 with sterile water, and prepare two litmus agar plates from it, using .5 c.c. for inoculation.

(*b*) Boil 100 c.c. in a flask for five minutes; cool, and prepare two litmus lactose agar plates from it as above.

(*c*) Plug the flask with cotton-wool and leave it in a room at ordinary temperatures for twenty-four hours; then prepare two litmus agar plates from it as before.

Incubate all the above at 35° C. for forty-eight hours; note and compare the number of colonies, their form and acidity, which appear on each plate.

Stain and examine specimens of the organisms from the different colonies.

(*d*) Place the unboiled milk remaining in a 100 c.c. flask by the side of the boiled sample.

Leave them until both are curdled, noting which curdles first, the nature of the curd, the separation

of whey, and any other features of difference between the two samples.

(*c*) **Pasteurization.**—The process of pasteurization has been extensively applied to milk with a view of increasing its keeping quality, without appreciably altering its natural flavour or nutritive property. It consists in heating the milk to a temperature of 60° C. to 80° C. (140° to 185° F.) for twenty minutes or less, and then cooling it rapidly to about 10° C. (50° F.). There is no definite standard of temperature for the process; that perhaps most generally adopted is 85° C. at which the milk is kept for three to five minutes. At 70° C. (158° F.), or lower, the heating should be continued for twenty minutes. In all cases for efficient pasteurization it is essential to keep the milk stirred or agitated so as to secure uniform heating.

The heating destroys from 95 to 99 per cent. of bacteria present in the vegetative state, and, if not too prolonged, does not give the milk a cooked taste. Cleaner milk is obtained, and at the low temperature to which it is immediately cooled the few remaining organisms cannot grow; the milk retains its freshness for three or four days, provided that it is kept in a cool place and free from dust and other contaminating substances. The lactic acid bacteria and many others are destroyed at the temperatures indicated above, but the spores and growing organisms of certain species remain uninjured. According to Flügge, the pathogenic organisms responsible for tuberculosis, diphtheria, and typhoid fever are killed by heating the milk to 70° C. (158° F.) for half an hour, although this treatment leaves it with a slightly boiled taste.

The great reduction in numbers by pasteurization is seen in the following table compiled from Professor Stewart's experiments :—

Average No. of bacteria per c.c. before pasteurization.	Time of Heating. Minutes.	Temp.	No. of bacteria per c.c., 24 hours after pasteurization.	Flavour.
136,262	10	60° C.	1722	Unaltered
78,562	10	65° C.	.58	Little affected
49,867	10	70° C.	0	Slightly boiled
77,062	10	75° C.	0	Boiled.

It is important to bear in mind that pasteurization is a double process, namely, the application of a temperature sufficient to destroy many living bacteria, followed by rapid cooling. To disregard the latter part of the process is to render it useless ; for if this is neglected, and the heated milk allowed to cool slowly, the remaining uninjured organisms multiply rapidly when the optimum temperature is reached, and the milk may contain more bacteria after treatment than before.

Ex. 142.—Take 250 c.c. of fresh evening's milk and keep it in the pantry until next morning.

Dilute a small amount of it to 1 in 1000 with sterile water, and prepare two litmus lactose agar plates, using .5 c.c. for inoculation.

Heat—(1) 50 c.c. of the milk to 50° C. for 10 minutes.
(2) ,, ,, ,, 60° C. ,, ,,
(3) ,, ,, ,, 70° C. ,, ,,

Cool quickly.

Dilute a small amount to 1 in 100, and prepare two litmus lactose agar plates from each sample, using .5 c.c. for inoculation

Incubate all at 35° C. for 48 hours.

Count the colonies on the several plates, and note the effect of the heating on the numbers of bacteria in the milk.

Observe which colonies are acid on each plate.

(iii) **Preservatives.**—The use of saltpetre, salt, and other substances for the purpose of preserving all kinds of food from putrefaction and other fermentative changes is widespread. Many attempts have been made also to check the souring of milk by the addition of certain chemical compounds which in small doses are known to inhibit bacterial growth. Such powerfully poisonous bactericides as mercuric chloride or carbolic acid cannot of course, be used for this purpose, but it is unfortunately too often the case that antiseptics with deleterious properties are added to milk by farmers and dairymen. The substances most commonly employed are boric acid, borax, salicylic acid, and formalin; in most countries their use is illegal, and infractions of the law should be punished.

All materials which effectually check the development of bacteria are certain to impair the digestive functions, particularly of infants and young children, whose diet consists largely of milk. There is little doubt that the continued use of small amounts of these compounds would result in damage to the health of adults also if milk were extensively used as a food by such persons.

The addition of small quantities by the farmer has been repeated by the dealer and dairyman, and in some instances by the householder as well, so that before the milk was consumed it contained pernicious doses of these harmful substances.

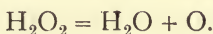
Apart from the directly injurious effects of preservatives upon the health of the community, their use

indirectly tends to foster negligence and uncleanliness in the production and distribution of milk, and prevents the spread of efforts to improve the conditions which prevail at the farms and dairymen's premises.

Although boric acid is, perhaps, not so injurious in small doses as formalin or salicylic acid, its addition to milk should be prohibited, and the law strictly enforced. These compounds do not preserve by eliminating or destroying the bacteria, and they are only used to make a dirty milk appear or behave as a clean sample.

For improving the keeping qualities of milk, it is best to depend upon cleanliness of the farm and dairy, and the maintenance of the product at a low temperature up to the time that it is needed for consumption.

During the last few years many investigations have been made upon the bactericidal power of hydrogen peroxide, a compound with somewhat poisonous properties, but which under certain conditions is readily decomposed into the two harmless substances, water and oxygen, thus :—



$$H_2O_2 = H_2O + O.$$

The free oxygen is given off in an active "nascent" state, in which condition it is able to destroy the vitality of bacteria and their spores.

The addition of small quantities to milk has been found to kill off the bacteria in it and render it sterile.

Its action is materially decreased by minute amounts of acids, and at low temperatures the quantity it is necessary to add to milk to destroy all bacteria present in it, is too great to allow of the milk being used for human consumption. However, at higher temperatures, very small quantities suffice for sterilizing

purposes. In Budde's process of sterilization, the milk is heated to 48° or 50° C., and to it is added .035 per cent. (about 12 c.c. of a 3 per cent. solution) of hydrogen peroxide. After keeping the milk at this temperature for half an hour, during which time it should be stirred or gently agitated, the temperature is raised to 52° C., and this degree of heat maintained for two or three hours. The milk is subsequently cooled rapidly, and sent out in sterile bottles.

The chief objections to the use of hydrogen peroxide for this purpose are these :—

(*a*) A very small amount of the compound remains undecomposed in the milk, and the effect of this upon the health of small children is not known with certainty.

(*b*) Comparatively minute quantities give the milk a taste which is disliked by some persons.

(*c*) Much of the hydrogen peroxide which is sold contains traces of hydrochloric acid, arsenic, and barium compounds, and the pure solution is too expensive at present for general use.

CHAPTER XIX.

THE EXAMINATION OF MILK: MILK STANDARDS.

1. **Dirt in Milk.**—A great many impurities are found in milk, some of them, as already noticed, consisting of inert matter not necessarily harmful; others, however, are of an objectionable or unclean character, and likely to damage its nutritive value, or cause more or less serious illness among those consuming it. Under present conditions of production and distribution, it is not possible to send to the consumer milk entirely free from pollution of one kind or another.

Much of the milk before it leaves the farm or dairy is passed through sieves or strainers of muslin, cotton-wool and similar materials, but these only remove the larger particles of foreign matter which fall into it at the time of milking or when it is in the cowshed.

The material collected by such strainers consists chiefly of hair from the cow, pieces of dried dung, and fragments of hay, straw, and other fodder. More rarely are found parts of dead flies and other insects, cobwebs, bits of feather and wood, woollen, linen, and cotton threads and fibres from the garments of the milker, skin from his hands, and hyphæ of fungi.

Although the grosser material is removed, a variable quantity of "dirt" passes through the ordinary strainers used, and appears as a sediment covering the bottom of

the vessels in which the milk is allowed to stand for a few hours. It may be more quickly collected, and its amount approximately estimated by means of a centrifuge.

Ex. 143.—Estimation of the sediment in milk.

Take a carefully mixed sample of the milk to be examined; not less than a litre should be used. Add 4 c.c. of formalin to it to prevent the growth of bacteria, and pour the milk into the apparatus (Fig. 48) up to 500 c.c. or the litre mark. Cover the opening of the cylinder with a glass plate, and allow the whole to stand for twelve hours. Push the rubber plug at the end of the brass rod through the milk to the bottom of the cylinder, so as to block the outlet at the bottom. Then remove the measuring tube and centrifuge at two thousand revolutions for three minutes. Remove the milk to just above the graduated portion of the tube with a pipette, and fill up to the 10 c.c. mark with a .1 per cent. solution of sodium carbonate; centrifuge again for two minutes.

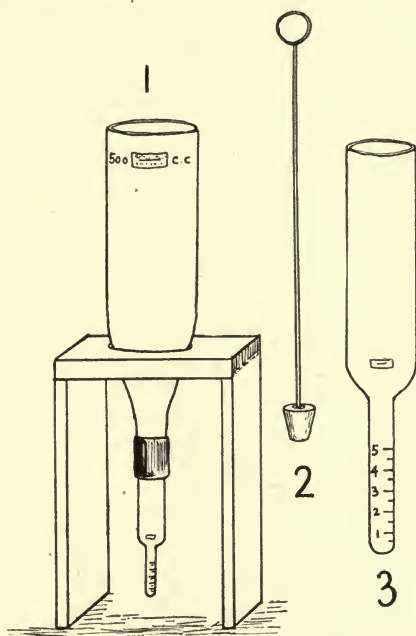


Fig. 48.—Apparatus for estimation of sediment in milk.

Read off the volume of the deposit obtained, and calculate the number of volumes of sediment in 1 million volumes of milk.

2. **Blood**; **Leucocytes**; "**Pus-cells.**"—Red blood corpuscles, and white corpuscles or leucocytes and "pus-cells" are not uncommon in milk.

The presence of blood may arise through temporary rupture of a small blood vessel in the udder, or may come from more serious lesions connected with disease of the gland. The red corpuscles in such cases may be sufficient in number to colour the milk. When present they may be easily recognized on microscopic examination of the sediment thrown down in the milk after it has stood for a time. They are circular bi-concave disks, without nuclei, from 6 to 8 μ in diameter, and of pale yellowish tinge when viewed singly.

On account of their form, light is unequally transmitted through them, the centre appearing brighter or darker than the surrounding border, according as the focus of the microscope is altered. When abundant they may be seen piled closely together in "rouleaux" like coins.

The colourless corpuscles or leucocytes are larger spherical protoplasmic structures, over 10 μ in diameter present in the blood and lymph of animals. They consist of granular protoplasm and contain one or more nuclei: in a living state they exhibit amœboid movement their outline constantly changing in form. Under certain conditions many of the leucocytes pass through the walls of the capillary blood vessels into the surrounding tissues; this is especially the case at points where there is inflammation. The pus or "matter" which accumulates in wounded or inflamed areas consists largely of dead, altered, or degenerate leucocytes, to which the term "pus-cells" has been applied.

The exact significance of leucocytes in milk is a matter upon which there is much difference of opinion.

"Pus-cells" cannot be distinguished at all times with certainty from normal leucocytes unconnected with inflammatory or diseased conditions. While it is certain that in the case of mastitis and other diseases of the udder, the number of leucocytes in the milk increases very considerably, it is not possible to fix upon a standard based upon number per cubic centimetre which shall be indicative of pus, since in milk from normal healthy animals the quantity is found to be subject to great fluctuation.

Usually the presence of many leucocytes accompanied by streptococci has been taken to denote suppuration or unhealthy inflammatory state of the udder, and milk in which both are found in abundance should be condemned as unfit for domestic use. The nuclei stain easily with methylene blue and are readily observed in stained cover-glass "films" prepared from sediment or centrifuge deposit obtained from milk containing them.

Ex. 144.—Examine microscopically the deposit obtained in the preceding Experiment.

First pipette off the sodium carbonate solution: then fill up with distilled water and centrifuge. Pipette off the water to near the top of the deposit. Stir up the latter with a platinum wire; remove a drop with a platinum loop and spread it as evenly as possible over a $\frac{3}{4}$ inch square cover slip.

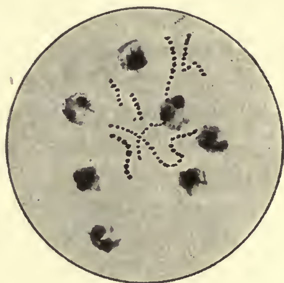


FIG. 49.—Leucocytes or cellular elements of milk, with *Streptococcus* chains.

Allow it to dry in a place free from dust and then immerse the cover in ether for three minutes: transfer to absolute alcohol for a minute, and after drying stain for ten seconds with Löffler's methylene blue.

Wash, dry and mount in Canada Balsam.

(*a*) Examine with a $\frac{1}{6}$ in. objective and No. 4 eye piece and count the number of leucocytes in ten separate fields (Fig. 49); calculate the average.

(*b*) Note the number and forms of bacteria present. Are chains of streptococci abundant?

(*c*) Prepare another film and after treatment with ether and alcohol, examine for presence of acid-fast organisms (see p. 325). Stain for several minutes with hot carbol-fuchsin, wash and decolourize with 25 per cent. sulphuric acid and counterstain for ten seconds with methylene blue.

Wash, dry, and mount in Canada Balsam, and examine with $\frac{1}{12}$ in. objective.

3. **Bacteria: number and kind.**—In addition to blood, pus-cells and inert substances, the milk contains various kinds of living bacteria, some of them derived from the interior of the udder, others being introduced in the particles of dung, fodder and other materials already enumerated.

The number of bacteria present at the time of delivery to the consumer is partly controlled by the amount of the initial contamination but is also dependent upon the temperature at which the milk has been kept, and the time which has elapsed since it was first drawn from the cow.

The fixation of a standard based upon the *number* of organisms is therefore difficult, and is of less moment than the examination of milk in regard to the *kinds* which it contains. Some of them render milk acid very rapidly, or otherwise alter it so that it soon becomes unfit for domestic use: others may set up decompositions which result in the formation of poisonous compounds or cause disease. An abundance of *Streptococcus lacticus*

would be less harmful than a similar number of *B. coli* or other organisms derived from dung, putrescent substances or sewage-polluted water.

The bacteria of the greatest importance from a hygienic point of view are the pathogenic species and those which indicate contamination with dirty water, sewage, fæces and similar objectionable materials. The examination of milk for the germs of disease is beyond the scope of this work. The exact and certain determination of the pathogenic character of many of them involves inoculation experiments upon animals, and no other method will give really reliable evidence of the presence or absence of *B. tuberculosis*.

(i) **Acid-fast Bacteria.**—It is however advisable to test milk for acid-fast organisms in the manner indicated in Ex. 150, and if found the further examination in other ways may be undertaken by the proper authorities if necessary.

(ii) **B. enteritidis sporogenes.**—As mentioned later (p. 330), Klein and others believe that *Bacillus enteritidis sporogenes* is responsible for epidemics of diarrhoea among children, but the matter is still in dispute.

Apart from this aspect, tests for *Bs. enteritidis* are useful, since it is a very common inhabitant of animal fæces and its presence may be taken as an indication of pollution for this source. According to Orr's investigation, it would seem that the farmer and not the dairyman is chiefly responsible for its occurrence in milk. As it does not multiply in milk except under anaerobic conditions its occurrence in 1 c.c. or less of any sample may be taken to denote unclean conditions at the farm.

Ex. 145.—Examine samples of milk for the presence of *Bacillus enteritidis sporogenes* by the method described in Ex. 151.

(iii) **Bacterium coli communis and allied organisms.** —The occurrence of *Bact. coli* in milk is one of the most important indications of pollution by manure, sewage or dirty water contaminated with fæcal matter.

The characters of the bacterium are given on p. 106.

Besides true typical *Bact. coli* there are a number of very closely allied organisms commonly met with in fæces and water which is polluted by animal excreta. They are spoken of as "coliform" bacteria, and are regarded as more or less permanent modifications or varieties of *Bact. coli*, differing only from the type in certain biological characters such as the power of curdling milk, fermentative action on various carbohydrates and production of indole.

For all of these forms a search should be made in milk: the following table, based on the admirable researches of MacConkey is of great value in diagnosing any organisms of this class which may be isolated.

CHARACTERS OF "COLIFORM" ORGANISMS.

	Motile.	Glucose.	Lactose.	Saccharose.	Dulcite.	Adonite.	Indole.	Voges & Proskr.
Bact. Grünthal	+	+	+	–	–	–	+	–
Bact. acidi lactici (Hüppe) .	–	+	+	–	–	+	+	–
Bact. coli communis	+	+	+	–	+	–	+	–
Bact. neapolitanus	–	+	+	+	+	–	+	–
Bact. oxytocus perniciosus . .	–	+	+	+	+	+	+	+
Bact. lactis aerogenes . . .	–	+	+	+	–	+	–	+
Bact. cloacæ	+	+	+	+	–	–	–	+
Bact. coscoroba	–	+	+	+	–	–	+	–

They all ferment glucose and lactose with the production of acid and gas; they acidify and throw down casein in milk in three or four days; none of them liquefy gelatine except *Bact. cloacæ* which induces liquefaction only when the cultures are kept for some time (four to thirty days).

Ex. 146.—To determine if acid and gas-producing organisms are in a sample of milk.

Prepare the following medium:—

MacConkey's Bile-salt Peptone Water.

Sodium taurocholate (bile salt) . .	2.5 gr.
Glucose	2.5 gr.
Peptone	10.0 gr.
Water	500 c.c.

Heat gently to assist solution of ingredients; filter, and then add sufficient amount of boiled Kubel-Tiemann's neutral litmus solution to colour the medium.

Pour it into Durham's fermentation tubes, 10 c.c. in each, and steam for twenty minutes on three successive days.

Inoculate some of these tubes with 1 c.c. of the sample of milk to be tested for acid and gas producing organisms; also inoculate with 1 c.c. of the milk diluted to $\frac{1}{10}$, $\frac{1}{100}$, $\frac{1}{1000}$, $\frac{1}{10000}$, $\frac{1}{100000}$, and $\frac{1}{1000000}$ (see Ex. 125).

Incubate at 37° C. for forty-eight hours.

Note in which dilutions acid and gas are produced.

Ex. 147.—Separation and identification of glucose- and lactose-fermenting (coliform) organisms in milk.

Prepare the following medium:—

MacConkey's Bile-salt Lactose Agar.

Sodium taurocholate	2.5 gr.
Peptone	10 gr.
Agar	7.5 gr.
Water	500 c.c

Heat in water bath for one hour, so as to dissolve the constituents and when cooled to 50° C. add the white of an egg, and heat again in water bath for 1½ hours; then filter. To the filtrate add:—

1 gr. of lactose	per 100 c.c. of the medium.
1 c.c. of a .5 per cent. solution of neutral-red	
1 c.c. of a .1 per cent. solution of "krystal-violet" . . .	

Pour the medium into sterile test-tubes, 10 c.c. in each, and sterilize by heating twenty minutes on three successive days.

Melt some of the tubes in hot water, and pour the contents into sterile Petri dishes.

After the medium is set, place the dishes in the incubator with the covers slightly open at one side, so that the surface may dry.

Then take a loopful of the cultures of the diluted milk of Ex. 146, in which both gas and acid production are observed, and mix it with 5 or 10 c.c. of plain sterile broth; from this mixture take out a loopful of the liquid and deposit it on the surface of the bile-salt agar in a Petri dish; spread it over the medium by means of a sterile glass rod, bent at right angles.

Incubate at 37° C. for forty hours.

Make sub-cultures of the different colonies observed upon ordinary agar slopes and incubate for twenty-four hours.

(i) Find if the organisms isolated are able to ferment glucose, lactose, saccharose, dulcite, and adonite by adding a loopful of the culture from the agar slope to Durham's fermentation tubes, containing the following broth:—

Lemco	10 gr.
Peptone	10 gr.
Sodium bicarbonate	1 gr.
Water	1000 c.c.

to which has been added 1 per cent. of the several carbo-

hydrates, and a small amount of boiled Kubel-Tiemann's litmus solution sufficient to colour the medium a violet tinge.

(ii) Apply the indole test to each (see Ex. 63).

(iii) Cultivate each in glucose bouillon and determine which gives the Voges-Proskauer reaction (see Ex. 65).

(iv) Cultivate each kind of organism in plain broth at 37° C., and, after six hours, examine a drop and determine which of them are motile.

(v) To which species does each belong; compare the results with the table on p. 306.

Ex. 148.—Determine the relative percentage of liquefying to non-liquefying kinds in several samples of ordinary market milk.

Dilute the samples to 1 in 10,000 if twelve hours old; to 1 in 100,000 if twenty-four hours old.

Inoculate two or three litmus gelatine plates with .5 c.c. of each, and incubate them at 22° C.

Observe the colonies from day to day; count the "liquefiers" and "non-liquefiers," and calculate the relative percentage of each.

Milk containing from 30 to 50 per cent. of liquefying organisms is suspicious and likely to have been contaminated with filth or dirty water.

Ex. 149.—Test the acidity of market samples of milk by the method described in Ex. 134.

How many fail to reach Newman's standard? (See p. 311.)

(100 c.c. of the milk should not require more than 22 c.c. of decinormal caustic soda solution for neutralization.)

4. **Milk Standards.**—From time to time various proposals have been made, chiefly at the instigation of the medical profession, for the adoption of standards of cleanliness, acidity, and bacteriological characters for all milk supplied to the public. In some countries such standards have been and are more or less rigidly enforced,

along with regulations framed to secure the sale of milk only from healthy cows, kept and milked under hygienic conditions. In the British Isles at present the matter is left largely to voluntary effort, and until the public can be educated to demand a sounder, cleaner milk, there will be no general improvement in the purity of this important article of diet.

Many investigations have been made to determine the amount of dirt, the acidity, and the number and kinds of bacteria in milk as it is actually delivered by the farmers, dairymen, and others who sell it; these researches are useful in giving a clue to the standard which might very well be adopted without hardship to the farmer or retailer of milk.

The imposition of certain regulations for the inspection of cows and cowsheds, and the fixation of legal standards of purity, all of which would be in the interests of public health, would undoubtedly involve expense to the producer and distributer, and this would have to be met by the payment of a somewhat higher price for milk than at present.

For farmers, dairy companies, and others who are desirous of supplying their customers with clean milk, the following standards should be kept in view:—

(i) **Dirt.**—As estimated by the method described in Ex. 143, market samples of milk have been found to contain from about 5 to over 120 volumes of dirt per million of milk. Most of the impurity is introduced at the farm, comparatively little being added during the journey to the consumer. From the work of Houston and Orr it would appear that the enactment of a regulation that in any sample *taken at the farm* in this country there should not be more than 30 or 40 volumes of

dirt per million of milk, would be a generous standard, and one which would not entail unnecessary and burdensome attention to cleanliness on the part of the farmer.

(ii) **Number of bacteria and temperature standard.**—Milk produced under clean conditions, if cooled to 10° C. or lower as soon as possible after it leaves the cowshed, and maintained at that temperature, need not contain more than 10,000 or 12,000 bacteria per c.c. in summer, or half this number in winter; some dairies are able to supply milk of this quality to their customers all the year round.

Park suggested that milk containing more than 100,000 per c.c. should not be sold for domestic use, but this standard is easily exceeded in districts where ice or other means of refrigeration are not available, especially in the warmer seasons of the year.

In Boston the standard of 500,000 per c.c. is enforced upon retailers, and milk for sale must be stored at temperatures not higher than 10° C.

In this country the number of bacteria in samples taken *at the cowshed* should not exceed 50,000 per c.c. Milk taken at the railway termini of large towns does not usually contain more than 500,000 per c.c., unless the weather is very warm, and the railway journey has been prolonged in exposed vans and waggons.

(iii) **Acidity.**—Milk which has not been kept cool, or which has been stored in unclean vessels in dirty shops, especially if obtained from a badly kept farm, soon becomes acid and more or less toxic from the action of "coliform" organisms in it.

Newman proposed that no milk should be sold 100 c.c. of which required for neutralization more than 22 c.c. of a deci-normal solution of caustic soda.

(iv) **B. enteritidis sporogenes.**—Milk found to contain this organism in 1 c.c. or less should be regarded with suspicion or condemned, especially if it is likely to be used for the feeding of infants and young children.

(v) **Glucose-fermenting bacteria.**—Where one-tenth of a cubic centimetre or less of a sample of milk, *taken at the farm*, is found to contain organisms which ferment glucose with the production of acid and gas, it should not be sold or used for domestic purposes.

CHAPTER XX.

SOUR-MILK BEVERAGES AND FOODS.

1. IN the manufacture of most alcoholic liquors, malt sugar or cane sugar are fermented by yeasts, alcohol, along with a certain amount of carbon dioxide gas and other substances being produced.

Lactose, or milk sugar, although a disaccharide, and similar in chemical composition to the sugars just mentioned, is not so readily fermented. A few yeasts are, however, known which are able to form alcohol and carbon dioxide from it, either directly, after hydrolysis, by their own "inverting" enzymes, or indirectly, after it has been decomposed into glucose and galactose by bacteria. Similar alcoholic fermentation can be accomplished by certain bacteria.

Generally, it would appear that the development of lactic acid organisms checks the fermenting activity of yeasts; but a few examples are known of the production of alcohol in milk soon after, or simultaneously with, the formation of lactic acid, the one process no doubt modifying the other.

In various parts of the world alcoholic beverages or foods containing lactic acid and alcohol are made from the milk of domestic animals by ferments, the chief of these being Kefir, Koumiss, and Mazun. Joghurt, a sour-milk product, containing little or no alcohol, must also be noticed here.

2. **Kefir.**—The name Kefir is given to an acid alcoholic beverage prepared from the milk of cattle, sheep, or goats by inhabitants of the Caucasus.

In the manufacture of it, milk is poured into skin bags or "bottles," and fermented by means of Kefir-grains. After two or three days, during which time it is shaken occasionally, it is ready for consumption, and forms a refreshing, foaming drink which, according to some authorities, possesses considerable medicinal and nutritive properties.

The chief chemical changes occurring in the milk are concerned with the milk-sugar. This constituent yields lactic acid, alcohol, and carbon dioxide. Casein is precipitated by the acid, and on shaking the liquid breaks up into fine flocculent particles which are readily digested.

The Kefir-grains, or active fermenting agents, in a dry state, are small yellowish tough bodies, about the size of large millet seeds or small peas. After soaking in milk or water, they swell up and grow into gelatinous, elastic convoluted structures which may become as large as a walnut.

They are found to consist of a zoogloea of yeast-cells and several kinds of bacteria which apparently carry on a symbiotic existence.

Many kinds of organisms are usually present, mostly as unavoidable impurities; according to Freudenreich, only three or four are essential for the characteristic fermentation of Kefir, namely, a non-sporing yeast or *Torula* (*Torula kefir*) met with chiefly in the rind of the grains, *Bacillus caucasicus*, and two forms of *Streptococcus*.

The yeast has roundish-oval cells measuring 3 to 5 μ by 2 to 3 μ, and produces a dark rose-coloured

growth on potato. It is incapable of inverting or fermenting milk-sugar by itself, but manufactures alcohol from the products of the hydrolysis of this sugar, which arise through the activity of a *Streptococcus b.* The latter is a small oval coccus which gives rise to lactic acid and gas in milk without precipitation of the casein. *Streptococcus a.* is a larger species, resembling *Str. lacticus* in many of its properties, but produces gas when grown in milk.

Bacillus caucasicus, Beijk. (*Dispora caucasica*, Kern). —One of the constant components of the interior of Kefir-grains is a non-motile organism 5 to 6 μ long, .4 to .5 μ broad, frequently curved or bent, and often having a spore-like granule at each end. It coagulates milk, forming much lactic acid but no gas.

Recently, Nicolaiewa states that she was able to produce good Kefir by the combined action of pure cultures of this bacillus and the *Torula*; the others she considers are impurities, as also *Torula ellipsoidea*, *Oospora lactis*, and *Sarcina lutea*, which are usually found in Kefir.

3. **Koumiss** is another alcoholic beverage prepared from milk. Its manufacture has been carried on from time immemorial by the nomadic tribes inhabiting the Steppes of Russia. These people make it from the milk of mares, asses, and camels.

To start the fermentation in fresh milk, a small amount of old Koumiss, or some of the dried sediment from an old sample is added; in some districts "barm," or "liquid yeast" from a brewery, is used for the same purpose.

The organisms at work in Tartarian Koumiss are not well known.

Some of the milk-sugar is fermented by a yeast, alcohol and much carbon dioxide gas being produced; at the same time lactic acid is formed, and the casein precipitated in a finely divided state. A portion of the casein is broken down into albumoses.

A good sample is a foaming, frothy beverage containing about 1 per cent. of lactic acid and not more than 2 per cent. of alcohol.

A form of Koumiss is manufactured in Europe from cow's milk by adding to it cane-sugar and beer-yeast, and allowing it to ferment for two or three days. This and similarly prepared beverages have been much recommended for the alleviation and cure of consumption, diabetes, and anæmia.

4. **Mazun.**—Mazun is a kind of coagulated fermented product made in Armenia and neighbouring countries from the milk of the buffalo, sheep, and goat, and occasionally from cow's milk. The milk to be fermented is boiled and when cooled down to about blood-heat a small amount of old Mazun is added; and the whole allowed to ferment for twelve to thirty-six hours.

The soured coagulum obtained is of variable consistency being thickest when buffalo's milk is used and thinnest when made from cow's milk.

It is eaten as a sort of junket, or can be mixed with water to form a piquant aromatic drink containing lactic acid and a small amount of alcohol.

The peculiar fermentation of Mazun, according to Weigmann, Grüber, and Hess, is brought about by the combined action of a yeast and two varieties of bacteria.

The yeast, a variety of *Saccharomyces pastorianus*, is able to ferment milk-sugar, and at the same time gives rise to a pleasant aroma.

The two bacteria to whose action lactic acid is due are, *Bacterium mazun* and *Bacillus mazun*, the former a non-motile organism producing much acid and a dense curd, the latter a slightly motile spore-bearing bacillus, which rapidly peptonizes casein when cultivated by itself in sterilized milk.

Mazun can be used for souring cream for the manufacture of butter, and a kind of cheese is sometimes made from the curd which it throws down in milk.

5. **Joghurt and Lactobacilline.**—Joghurt (pronounced Yoh-oort) is a form of acid-coagulated milk used extensively in Turkey, Bulgaria, and the Northern Balkans.

The milk of the buffalo, cow, sheep, or goat is thoroughly boiled for a time, and after cooling to 45° C. is inoculated with an old sample of Joghurt or some of the "ferment" obtained from it, which is named "Maya." It is then kept at a temperature of about 40° C. for nine to sixteen hours when the fermentation is complete.

The thick coagulated milk which has an agreeably acid taste and a pleasant aromatic odour, is then eaten either alone or with bread, rice, or other foods.

Genuine Joghurt, obtained from districts where it is in daily use, usually contains a considerable number of different bacteria, yeasts, and moulds, many of them being unavoidable impurities.

The characteristic organism is *Bacillus bulgaricus*, Heupel, which according to Luerssen and Kühn is frequently accompanied by two other bacteria briefly described below.

In the fermentation lactic acid is formed in abundance with little or no alcohol, on which latter account this product appears to be more beneficial than Kefir or Mazun.

During the last few years a great deal of attention has been paid to this and similar acid-milk pro-

ducts on account of their supposed dietetic and remedial value.

Several hardy, long-lived races in different parts of Eastern Europe and Western Asia consume considerable quantities of fermented milk, and it has been assumed that their freedom from disease and longevity may be directly connected with the use of this article of diet. In the intestinal tract of man, especially in the colon, putrefactive bacteria are abundant. Here they carry on their work of decomposition and give rise to indole, skatole and other foul-smelling, poisonous compounds. According to Metschnikoff these are absorbed into the system and bring about premature old age.

With a view of checking or reducing such intestinal auto-intoxication and changing the bacterial flora of the colon, he has recommended the introduction of the lactic acid organisms through the medium of sour milk.

As it is not safe to consume spontaneously-soured samples on account of the possible contamination with disease germs, pure cultures of suitable lactic bacteria in sterilized or pasteurized milk are used instead.

Fig. 50.—The Joghurt bacillus (*Bacillus bulgaricus*, Heupel).

The chief organism employed for this purpose is *Bacillus bulgaricus*, Heupel, which has been isolated from Joghurt or Maya by Massol, Grigoroff, Luerssen, Kühn, and others (Fig. 50).

Pure cultures are on the market in the form of tabloids and powders, and sold under various names, such as "Lactobacilline," "Joghurt powder," and "Maya ferment."

Attempts have also been made to introduce them into chocolate and other bon-bons.

Much care, however is needed in the preparation of pure cultures on a commercial scale, and many of these products, for which absurdly exaggerated claims have been made, are found on examination to be impure, or to contain few of the right organisms in a living condition.

The Joghurt bacillus (*Bacillus bulgaricus*, Heupel), is a long, slender, rod-shaped bacillus, rounded at the end, occurring singly or in chains and threads. Single cells are usually about 3 to 10 μ long, and 1 to 1.25 μ broad. It stains by Gram's method, is aerobic or anaerobic, and difficult to cultivate. It grows best at 45° to 50° C., little or no growth at 20° C., slow at 25° C., fair at 30° C.

Whey gelatine.—Stab, filiform, beaded, not liquefied, and no surface growth.

Whey agar.—Colonies roundish or irregular, greyish, .5 to 1.5 in diameter, with filamentous curly margins.

Potato.—No growth.

Milk is rendered acid and coagulated in eight to eighteen hours at 44° C. There is no gas formation, and the casein is not peptonized.

This organism is remarkable in being able to produce large amounts of lactic acid in milk, some forms giving rise to over 3 per cent. in ten days when incubated at 35 to 36° C.

The *Granular bacillus of Joghurt* is a longer organism, which stains irregularly with Löffler's methylene blue. Its surface colonies on sugar agar media are transparent and irregular; deep colonies, curly or radiate. It grows best at 37 to 45° C.

On potato no visible growth occurs.

Milk is coagulated and becomes acid very rapidly at 37 to 40° C.; no gas formation.

The Streptococcus of Joghurt occurs usually in pairs, which stain readily with Gram's gentian violet. It grows only on milk agar, in small, yellowish-white, round colonies. Milk is made acid and coagulated.

CHAPTER XXI.

MILK AND DISEASE.

1. A GREAT many of the epidemic diseases to which mankind is subject have been spread by means of water and milk. Fortunately, during the last century vast improvements have taken place in the selection and management of the public water supply: purer sources have been tapped, and better regulations framed and carried out in regard to the storage and distribution of water for domestic purposes; the disposal of sewage has been supervised, and contamination of drinking water by disease germs has been very greatly reduced.

A different picture is presented to view when the question of the relationship of the milk-supply to disease is examined, and much remains to be done, both by the farmer and the general public, before the standard of purity and safety is as high for milk as for water.

Milk is one of the most important of all foods, especially for the young, and, unlike most of the materials consumed, is very largely taken into the system in a raw, uncooked, and unsterilized state.

Moreover, it is a liquid which is eminently adapted for the nutrition of many kinds of bacteria, and disease producing organisms which gain access to it, grow and multiply in it at an enormous rate. Such infected milk has not infrequently spread illness and death over a wide area in a short time.

Milk-borne diseases may be divided into two groups, namely :—

(1) Those which attack both the cow and man, such as tuberculosis, foot and mouth disease, and possibly other illnesses ; and

(2) Those which are rarely or never met with among dairy stock, but whose germs find their way into the milk after it is drawn : to this class belong typhoid fever, diphtheria, scarlet fever, and certain obscure throat troubles, diarrhœa, and intestinal ailments.

2. **Tuberculosis.**—One of the great scourges of the human race is tuberculosis. The number of deaths per annum in the British Islands due to various forms of the disease is over 60,000, and probably more than ten times this number suffer from it.

The disease is also found extensively distributed among domestic animals, being specially prevalent in cattle and pigs ; less of the trouble is met with in horses, sheep, and goats. Cats and dogs are subject to it, and a form of the disease is common among poultry.

There are from 2½ to 3 millions of cows and heifers in Great Britain ; about 25 to 30 per cent. (600,000 to 800,000) are tuberculous ; according to M'Fadyean about 2 per cent. of them, representing a total of over 50,000 cows, have the disease in the udder, and yield milk containing the virulent germs of the disease.

In Islington (London) Dr Harris found, in 1899, over 14 per cent of the samples of milk examined contained the tubercle bacillus ; from 28 to 38 per cent. of the milk examined in Berlin in 1897 by two different observers was found to be infected, and in Paris 33 per cent. of the samples gave similar results. The undoubted presence of these bacilli in the milk supplied to

the public makes the question of the transmissibility of the disease to mankind through the consumption of this article of diet a matter of first-rate importance.

A great amount of research has been made into the relationship between bovine and human tuberculosis, the mode of infection of animals and man, and the morphological and physiological variations of the bacilli derived from various sources; many of the points raised in the investigation of these problems are still unsettled.

Koch, the discoverer of the bacillus, put forward, in 1901, the view that the organisms responsible for human and bovine forms of the disease are distinct, at any rate in infective power, and maintained that the bacilli of tuberculosis in cattle are very rarely, if ever, responsible for the disease in man.

Of course definite experimental proof by inoculation is out of the question, but the Royal Commission appointed to investigate the problem, although its final conclusions are not yet forthcoming, has obtained sufficient reliable evidence to confute Koch's statement.

It has been found that the bacilli derived from bovine tuberculosis infect calves, pigs, cats, dogs, the Rhesus monkey, and the chimpanzee, when inoculated into the system or supplied to the animals in food; they are therefore not specially adapted to attack cattle only. The experiments have also shown that the bacilli derived from human sources are able to produce the disease when given in the food or introduced by inoculation into calves, rabbits, monkeys, and apes.

The Commission has already reported that: "There can be no doubt that in a certain number of cases the tuberculosis occurring in the human subject, especially in children, is the direct result of the introduction of the

bacillus of bovine tuberculosis, and that in the majority of these cases the disease is introduced through cows' milk."

The dung and other excreta of tuberculous animals frequently contain the bacilli of the disease, and in a dried form is found floating in the air of cowsheds, barns, and other farm buildings. Infection among cattle is accomplished through the inhalation of dust of the infected premises or of bacilli coughed into the air by diseased animals, and also through the feeding of calves with milk containing the organisms.

In Denmark and some other countries the separated milk sent back from creameries to the farms for the feeding of calves and pigs must first be pasteurized, so as to destroy any virulent bacteria in it; without this precaution the milk from infected cattle on one farm may be carried to another which is free from the disease.

Tuberculosis is spread among human beings chiefly, perhaps, by organisms coughed into the air by consumptives and the bacilli of dried sputum, which rise with dust into the air and are breathed into the lungs. Flügge and other authorities consider that the disease is conveyed to children mainly through the digestive tract in milk and other food containing the bacteria, some holding the view that tuberculosis of the lungs or phthisis frequently arises from organisms entering the system in this way.

The tubercle bacillus gains an entrance into milk chiefly from cattle with tuberculous udders, but infected milk may be given by cattle in which the disease cannot be detected in the milk glands. It also enters in the form of cowshed or stable dust, consisting of particles of dung and other excreta from diseased animals. Possibly

consumptive milkers may sometimes be responsible for the infection of the milk.

It is only to be expected that butter, cheese, and other dairy products prepared from unsterilized milk or cream will not infrequently contain tubercle bacillus: such has been found to be the case by many different workers. Moreover, the organism may live in milk for several months, and in cheese for a year or more.

To avoid danger from these sources it is advisable, wherever possible, to manufacture dairy products only from milk or cream which have been pasteurized.

Bacillus tuberculosis (R. Koch), is a very variable organism. It is non-motile, usually rod-shaped, from 3 to 5 μ long, .5 μ broad, sometimes curved, and in old cultures frequently appears in the form of branching threads.

In stained specimens a number of unstained vacuole-like portions are usually seen.

It stains easily with carbol-fuchsin and the stain is "*acid-fast*" —*i.e.* it is not decolourized by 25 per cent. sulphuric or 30 per cent. nitric acids: it also stains by Gram's method.

The bacillus is aerobic, and thrives best at 37° C. Its thermal death-point is of great importance in regard to the destruction of living organisms in milk by the process of pasteurization. Many experiments have shown that temperatures of 60° C. maintained for twenty minutes, 65° C. for fifteen minutes, or 70° C. for ten minutes, are usually sufficient to destroy it, but Bang considers that nothing less than 85° C., kept up for five or ten minutes, suffices for absolutely reliable results.

Direct sunlight destroys the bacillus very soon, and diffuse daylight acts in a similar way but more slowly.

A 5 per cent. solution of carbolic acid kills it in thirty to sixty seconds, absolute alcohol in five minutes and a 1 in 1000 solution of mercuric chloride in ten minutes.

Growth in artificial media is always slow, and fortunately little or no growth occurs in milk.

Glycerin Agar.—Bs. tuberculosis grows scantily or not at all on ordinary agar or gelatine. On glycerine agar the growth in ten to twelve days is small and crumbly; later it extends, and in six or eight weeks may form a wrinkled lichen-like patch, whitish-yellow or brownish-yellow in colour.

Potato.—It grows in four or five weeks on potato placed in a Roux's tube and kept moist with five per cent. glycerine; the growth is dull and yellowish.

Veal broth containing 5 or 6 per cent. of glycerine is a good medium for it. Small cream-coloured flocculent masses are produced in the liquid with surface film-formation occasionally.

Ex. 150.—Examine a sample of milk for "acid-fast" organisms.

(*a*) Half fill two centrifuge tubes with milk and rotate for five minutes at 2000 revolutions per minute.

(*b*) Break up the cream and centrifuge again.

(*c*) Remove the cream and pipette off the milk to just above the deposit. Add water so as to half fill the tubes and rotate for five minutes.

(*d*) Pipette off the water, and transfer a drop of the liquid and the deposit with a platinum loop to a cover-slip. When air-dry pass through flame.

(*e*) Determine if any "acid-fast" bacteria are present. To do this take the cover-slip and place upon it 2 or 3 drops of weak carbol-fuchsin (see Ex. 8). Gently heat to steaming-point (60° C.) over flame for three minutes with aid of Cornet forceps, adding fresh stain to supply the place of that which evaporates. If preferred, the films may be heated while staining over an asbestos board or copper plate, or floated in a watch-glass containing the stain.

(*f*) Wash well in water and then immerse in a 25 per cent. solution of sulphuric acid for twenty seconds: wash in water, and if pink colour comes out repeat the treatment with acid and

water alternately until no more colour washes out when placed in the latter.

(*g*) After decolourization stain for one minute with Löffler's methylene blue and wash well: dry and mount in Canada balsam.

(*h*) Examine with $\frac{1}{6}$ and $\frac{1}{12}$ in. objective: the acid-fast organisms and spores of certain species which may possibly be present appear red, other bacteria, pus and mucus are blue.

Methods of checking or stamping out bovine tuberculosis.—It is outside our province to discuss the question of the methods to be adopted to deal with human tuberculosis; the farmer, however, should endeavour to stamp out the disease from his herds and make an effort to prevent tuberculous milk from being supplied to his patrons.

(i) All animals suffering from disease of the udder, and "wasters" in an advanced state of disease, should be destroyed immediately in order to prevent them from infecting the healthy stock.

(ii) Much good can be done by isolation and subsequent elimination of all animals which give evidence of tuberculosis when tested with "tuberculin": the test should be applied at intervals for some considerable period.

Two kinds of *tuberculin* are in use, namely: (1) the *old*, a filtered extract from a glycerine veal-broth culture of *Bs. tuberculosis*; (2) the *new*, a centrifuged extract of a dried and thoroughly pulverized culture.

When injected in very small quantities into the blood of an animal suffering from tuberculosis, even in a clinically unrecognizable form, there is a very marked rise of 1.5 to 2.5° C. in twelve to fifteen hours.

Healthy animals when treated with tuberculin are not affected by it.

(iii) Milk from suspected animals, or those reacting to tuberculin, should be carefully pasteurized or sterilized before being used for the feeding of calves or other farm stock, for the tubercle bacillus is sometimes present in milk from cows whose udders are apparently free from the disease; and where tuberculosis is present in the system it may develop and involve the udder at a rapid rate.

Rabinowitsch, Kempner, and Mohler showed that from 20 to 50 per cent. of the cows which reacted to tuberculin gave milk containing virulent bacilli capable of setting up tuberculosis when inoculated into animals, although the reacting cows showed no signs of udder disease.

(iv) Separated milk from creameries and dairies generally should be pasteurized before being used.

(v) Only animals which do not react with tuberculin should be retained for breeding purposes.

(vi) Good ventilation and adequate lighting of the buildings in which cattle are housed tend to reduce the disease; damp, dark, badly ventilated sheds reduce the vitality of the animals and render them less resistant to the attacks of the tubercle bacillus.

3. **Foot and mouth disease** of cattle may be conveyed, especially to infants and young children, by infected milk, but occurrences of the disease among human beings are comparatively rare.

4. **Typhoid fever.**—Many epidemics of this dangerous illness have been traced to the consumption of milk containing the typhoid bacillus (*Bacillus typhosus*). The disease does not attack cattle, but the entry of the

organisms responsible for it takes place through contaminated water used at the farm or dairy.

The bacilli, which very closely resemble *Bact. coli*, are found in the blood and also in vast numbers in the fæces and urine of typhoid patients. Even after recovery in some cases virulent organisms continue to be discharged in the excreta for months or years, and to such apparently healthy typhoid carriers are attributed the mysterious outbreaks and recurrences of the disease which are recorded from time to time.

At cottages, farms, and dairies wherever the sanitary arrangements are unsatisfactory, and the thorough disinfection of excreta from typhoid patients is not carried out, there is great danger of the wells or other sources of the water supply becoming contaminated with living typhoid germs.

The use of such polluted water for the washing of churns, milk-pails and other dairy utensils is the chief means of introducing the bacilli into milk, and numbers of outbreaks of the disease have been proved to originate in this way.

There is evidence also to show that flies after settling upon fæces containing typhoid organisms often carry upon their head, feet, and wings the germs of the disease, and transfer them to milk and food to which they have access.

Dry infected excreta may blow about as dust and find its way into milk.

B. typhosus thrives and multiplies in milk for many days, but its development is considerably checked by the lactic organisms; Bassenge states that an acidity of .4 or .5 per cent. destroys the bacillus in twenty-four hours.

Pasteurized and boiled milk constitute good media for the growth of the bacillus, and when introduced into these, the organism, having a field free from competition, grows rapidly and remains virulent for a month or more.

It is, therefore, very necessary to use clean vessels for storing pasteurized or boiled milk, and cover them so as to prevent access of flies and dust, which might reinfect the milk.

In cream the typhoid bacteria become more abundant than in milk, especially if the acidity is abnormally low, and butter prepared from it is infective.

The time during which they remain in a living or virulent condition in butter appears to depend on the acidity of the cream from which it is made ; in ordinary acid-cream butter they probably die out in a week or ten days.

5. **Epidemic Summer Diarrhœa** is one of the most fatal ailments to which infants are subject, the death-rate among children up to five years of age from this disease alone amounting generally to over 10 per 1000 ; its elimination would very materially alter the vital statistics of young children.

The fatality from this disease is highest in the warmest part of the year, namely, in the months of July and August, and is especially prevalent among children of the working classes in urban areas.

Many investigations have shown that it is essentially a trouble connected with the use of cows' milk, hand-reared infants being most affected by it.

The disease is apparently due to poisonous compounds in the milk, manufactured by swarms of bacteria introduced from dirty surroundings, the production of the deleterious substances taking place either before the milk is taken or after it enters the stomach and intestinal tract.

Hitherto no single specific organism has been found to which the illness can be attributed with certainty; possibly the bad effects are brought about by the combined action of two or more kinds.

There is little doubt, however, that the responsible organisms are introduced in dust and dirt.

Booker came to the conclusion that the species most actively concerned with the disease are *Proteus vulgaris*, and a *Streptococcus*, probably *S. pyogenes*, although Klein attributes the complaint to *Bacillus enteritidis sporogenes*, a widely-distributed anaerobic organism found in soil, sewage-polluted water, and fæcal matter, and frequently present in milk.

Careful cooling and scrupulous cleanliness in the production and distribution of milk in the summer, along with care in the use of clean vessels for storage of milk at home, would very greatly reduce infant mortality from this cause.

Bacillus enteritidis sporogenes, Klein.—This is a common anaerobic spore-producing organism isolated originally from human excreta and milk, the consumption of which had led to an epidemic of diarrhœa. It is frequently found in soil and in animal fæces.

The bacilli are 2.5 to 3.5 μ long, .8 to 1.25 μ broad, sometimes motile, and readily stain by Gram's method. The spores are oval, usually placed in the middle of the bacterial cell. It very closely resembles *Bacillus Welchii* and the non-motile butyric acid bacillus (p. 288).

Gelatine.—Surface colonies, semi-transparent, no liquefaction. In stab-cultures the colonies are small and spherical, and many gas bubbles are produced.

Agar.—Surface colonies, round, grey, flat, with thin lobed or indented margins.

Milk.—The changes in recently boiled milk, inoculated with this organism and cultivated anaerobically, are characteristic. Much gas is formed; the casein is coagulated and the surface is covered with pinkish scum enclosing gas bubbles. The whey below is clear and colourless, has a strongly acid reaction and rancid smell, butyric acid being produced.

Ex. 151.—Test samples of milk for *B. enteritidis sporogenes*:—

(i) Add 10 c.c. of the milk to a sterile tube; plug and heat at 80° C. for 15 minutes. Place the tube in a wide Buchner tube (Fig. 25) containing pyrogallic acid and caustic potash; seal so as to secure anaerobic conditions and incubate at 37° C. for two days. Pinkish frothy scum appears if *B. enteritidis sporogenes* is present.

(ii) Try the same experiment, using 20 c.c. of milk.

(iii) Try the same experiment, using 1 c.c. of the milk, adding it to a tube of sterile milk.

6. **Diphtheria and Septic Sore Throats.**—Several outbreaks of true diphtheria have been traced to the milk-supply, and in the majority of instances there has been definite evidence to show that the bacillus of the disease found its way into the milk from human sources and did not come from the cow. At the farm or dairy supplying the milk one or more persons were suffering from diphtheria or had only recently recovered from it. In many cases of mild throat epidemics similar evidence of the human infection of the milk is generally forthcoming.

There are, however, many instances where epidemic "septic" sore throats of a more or less diphtheritic character have been set up by milk given by cows suffering from various obscure forms of mastitis or inflammatory diseases of the udder

The exact nature of these illnesses, their cause, and the relationship to the milk-supply are still obscure.

7. **Scarlet Fever.**—From its infectious character and the peculiar course of its development it is inferred that scarlet fever is due to a minute, but still undiscovered, organism. The disease is disseminated very readily from one person to another, and by means of linen, bed-clothes, books, letters, and other objects which have been in contact with patients suffering from it. Without doubt milk can carry the contagion, and a great many epidemics of the disease have been spread by infected milk. In most of these cases it has been found that the milk received its disease-producing power from human sources, either the convalescent milkers or some one employed about the farm or dairy suffering from the ailment supplying the virus.

Some authorities, however, entertain the view that cows may suffer from some form of the disease, and convey the contagion to the milk.

CHAPTER XXII.

CREAM AND CREAM RIPENING.

Cream.—When milk is placed in a shallow vessel and allowed to stand for a time, the globules of fat, which have a smaller specific gravity than the serum in which they are distributed, rise to the surface of the liquid and form a layer of variable thickness. This layer of fat globules, with a certain amount of milk serum, is known as *cream*; it can be poured off or removed in other ways, leaving behind skim-milk, a product comparatively poor in fat.

In addition to this method of obtaining cream, which may take several hours to complete, cream may be taken from fresh milk by the centrifugal separator in a few minutes.

The composition of cream is very variable: thick or thin cream can be obtained at will by means of the separator, and the amount of serum left in it even when raised by gravitation processes can be regulated to some extent by "raising" the cream in shallow or deep vessels and varying the temperature at which the milk is kept. The comparative composition of cream, "separated," and fresh milk is shown below:—

			Per cent.		
	Water.	Fat.	Proteins.	Milk-Sugar.	Ash.
Thin cream	64	28	3.25	4.5	.65
Thick ,,	44	45	3.05	2.7	.60
Separated milk	90	.1	3.9	4.9	.76
Skim-milk (shallow pans)	...	1-1.5	...	...	...
Fresh milk	87	3.9	3.4	4.75	.75

The bacterial content of fresh cream obtained by means of the separator is usually low, especially if the milk has been recently drawn from the cow under clean conditions. It is generally lower than that of the milk from which it is derived, since many of the bacteria in the milk are driven to the sides of the separator bowl, and are collected in the protein "slime" along with any other solid particles which may happen to be present.

Cream of this kind is sweet, bacteria having had no time to develop or bring about any change in it. In fresh cream raised in deep or shallow pans in twelve to eighteen hours by gravitation, bacteria are found in considerable numbers. The organisms occurring in the milk when it is "set" soon multiply, and by the time the cream is a day old the numbers present vary from about 250,000 to over 6,000,000 per cubic centimetre.

Although sweet, freshly-separated cream is sometimes churned and made into butter, the greatest amount of butter is made from cream which has been kept from thirty-six to forty-eight hours and allowed to become sour either spontaneously or after the addition of special cultures of bacteria. This "souring" process is in reality a complex kind of fermentation termed "*ripening*."

In the ripening of cream certain chemical and physical changes occur, and there is a very extensive multiplication of bacteria.

Cream properly ripened and ready for churning possesses a clean acid taste due to the lactic acid fermentation; the amount of acid allowed to develop before churning should not exceed .5 or .65 per cent. calculated as lactic acid.

In addition to the acid taste, ripening results in the production of a characteristic taste or flavour of butter

and a pleasant odour or aroma; to what these latter characters are due is not known with certainty. Since it is found that lactic bacteria alone cannot properly ripen cream, it is probable that aromatic compounds of an agreeable flavour are produced by the action of bacteria allied to the putrefactive organisms upon the proteins of the serum, these being absorbed by the fat and transferred ultimately to the butter.

Very little, if any, chemical changes occur in the fat, but its physical characters are altered by ripening and ripened cream churns more quickly than sweet cream; it also gives up more of its fat in the form of butter, and less of the fat remains in the buttermilk than when sweet cream is used.

The exact nature of the change in the fat which enables the globules to coalesce readily when shaken in the churning process is not understood.

Some authorities have put forward the view that the globules are surrounded by a film of protein, which is dissolved or decomposed during ripening, thus allowing easy amalgamation of one globule with another. It is, however, more likely that the phenomenon is connected with alterations in the viscosity and surface tension of the serum.

Ex. 152.—Mount a drop of cream on a slide, and examine with a $\frac{2}{3}$ and $\frac{1}{6}$ in. objective.

Compare the number and appearance of the fat globules with those of a similarly mounted drop of skim-milk.

As previously mentioned, unripened cream contains from $\frac{1}{4}$ to 6 millions of organisms per c.c.; in the ripened product from thirty-six to forty-eight hours old, at which period the bacteria reach their maximum develop-

ment, from 500,000,000 to 1,500,000,000 are present; few other natural liquid media contain so many.

After reaching this point the numbers decline with the age of the cream; in four or five days a few millions only are found, and at the end of a week nearly all living organisms have disappeared except the fungus *Oospora lactis.*

There is thus a periodic rise and fall in the number of bacteria in cream similar to that which has been observed in milk when it is kept.

In freshly-raised cream there is a miscellaneous collection of different kinds of bacteria. Liquefying varieties are common, as well as others which have little apparent action on milk or gelatine media; lactic acid forms are comparatively rare.

During the first twenty-four hours after the milk is drawn from the cow the growth of all the kinds goes on simultaneously, and large numbers are carried by the upward stream of fat globules into the cream layer after the milk has been "set" for a time. About 2 to 20 per cent. are liquefiers; 10 to 75 per cent. neither produce acid nor liquefy gelatine; the rest are lactic acid varieties.

As ripening proceeds the latter multiply enormously, until when the cream is thirty-six to forty-eight hours old they form 95 to 99 per cent. of the many millions present; all other varieties decrease to very small proportions, or die out altogether.

Of the lactic acid bacteria found in ripened cream, *St. lacticus* and a nearly allied form are far the most abundant. *Bact. lactis aerogenes* is also invariably met with at all stages of the process, but is never found in large numbers when ripening runs its normal course.

Later the lactic forms vanish also, and only fungi remain.

Ex. 153.—Make a careful study of (1) the numbers and (2) the kinds of bacteria in—

(*a*) Freshly separated cream.
(*b*) Well-ripened cream, thirty-six to forty-eight hours old.
(*c*) Cream left in a covered vessel away from dust for ten days.

Dilute and plate out on litmus lactose gelatine and agar media as described in Exs. 125, 126.

What percentage produces acid or liquefies gelatine?

Examine and prepare permanent stained slides of the various colonies found.

Grow pure cultures of the different kinds of organisms on agar slants, and

(i) Test the action of each upon :—
(*a*) Milk.
(*b*) Nitrate bouillon.
(*c*) Bouillon containing the various carbohydrates mentioned in Ex. 147 (i).
(ii) Test their power of producing indole (Ex. 63).
(iii) See if any give the Voges and Proskauer reaction (Ex. 65).

Classify and, as far as possible, name the organisms found, comparing them with authentic examples.

The ripening process is one of the greatest importance from the dairyman's point of view: when it goes wrong the butter frequently turns out to be objectionable in flavour and aroma, and of poor keeping quality. Endeavour should, therefore, be made to control it, and although want of knowledge of many of its chemical and bacteriological details makes exact direction impossible, much can be done in a general way to guide it.

Temperatures between 18.5° and 21° C. (65° to 70° F.)

have a salutory influence upon it. Below about 18.5° C. (65° F.) the beneficial lactic acid bacteria are checked, and the organisms likely to give rise to bitter flavours and offensive odour are assisted: above 21° C. (70° F.) gas-producing forms often flourish, and the cream and butter become "oily."

Appearance and smell will sometimes guide an experienced buttermaker in determining the ripeness of the cream, but it is generally advisable to determine by chemical means the degree of acidity acquired, for it is found that when the cream contains .5 to .6 of acid (calculated as lactic acid), the process has usually advanced as far as it is advisable to allow it to go. Care should be taken to prevent the acid from becoming greater than this, or precipitation of the casein in lumps takes place, and these appear later as white specks in the butter and seriously deteriorate its flavour and keeping quality. Acidity in itself is not necessarily an accurate gauge of correct ripening for acid products, and the development of agreeable flavour and aroma are different and distinct features of the process: nevertheless, under normal conditions experiment has shown that the degree of acidity mentioned is generally correlated with suitable ripeness for butter-making purposes.

At the temperatures indicated above ripening should be complete in one or two days in summer and three or four days in winter.

Encouragement should be given to the aerobic bacteria by frequent stirring of the cream so as to ensure an adequate supply of air throughout the liquid; bad flavours are frequently associated with exuberant growth of anaerobic species.

The kinds of bacteria which develop in the ripening cream are of primary importance in the manufacture of good butter, since the preponderance of a single prejudicial species in it will often ruin the flavour and cause financial loss. As already pointed out, in normal ripening there is a succession of waves of growth of different kinds of bacteria, and hitherto no complete solution has been obtained of the problem of how to secure the presence of the right kind of organism at the right moment in adequate numbers. No single species isolated from cream has been found to be productive of completely satisfactory results when introduced and grown in sterile cream.

Tentative methods which go a long way towards effective control of ripening are in use; these consist in the addition to the cream of what are known as "*starters*," which are more or less pure cultures of one or two varieties of bacteria.

Two classes of starters may be recognized, namely:—(1) *natural starters* and (2) *artificial* or so-called "*commercial*" *starters*.

Natural Starters consist of milk or cream which has been allowed to turn sour and ripen spontaneously; buttermilk from a dairy which is producing butter of fine flavour and aroma may be included in this class.

For the preparation of these home-made starters the following procedure may be adopted. Select a healthy cow in the herd, and after washing the udder and flanks of the animal with soap and warm water, draw the milk, rejecting the first few jets, into a clean pail which has been sterilized by boiling water or steam. Cover up the vessel to prevent the access of dust, and keep it in a warm place, at a temperature of 65° to 70° F. in summer,

75° F. in winter, for twenty-four to forty-eight hours, until it has become sour but not curdled.

The development of more than .6 to .7 per cent. of acid should be avoided as likely to weaken the useful lactic bacteria, and give an opportunity for the butyric and putrefactive types to grow.

After removing the cream from the surface the skim-milk may be used to inoculate the cream to be ripened. If a larger quantity of starter is needed, that already prepared in the above manner should be mixed with the requisite amount of separated milk, which has been pasteurized at a temperature of 180° F. for half an hour, and the whole then allowed to sour. Some prefer not to use the spontaneously soured milk directly for inoculation, but the second or third subcultures of it in pasteurized milk.

In any case starters of this kind are the result of chance, and are not always satisfactory; bad taints difficult to get rid of may be propagated by them. Trial alone will settle whether a sample prepared in this way is good or bad.

Ex. 154.—Prepare a home-made starter by the method just described.

Plate out and determine the kinds of organisms in it.

Try their action upon milk separately.

Pure Culture Starters.—With a view of reducing failures to a minimum many attempts have been made to prepare pure cultures of bacteria which, when introduced in sufficient numbers into cream will lead to good uniform ripening.

Very many kinds have been isolated from milk, cream, and other dairy products and tested from this point of

view, and although no single species has been discovered to possess all the qualities for the production of the highest grade of butter, several varieties have been found which are highly beneficial in the preparation of well ripened cream. Single pure cultures of these, or mixtures of pure cultures of two or more kinds, have been placed on the market, and large quantities of such commercial starters are extensively used wherever dairying is practised.

Jensen suggests that for reliable and satisfactory results a culture should possess the following properties:—

a. It should render cream sour in a short time;

b. The organisms in it should be able to grow at a temperature of 60° to 65° F.;

c. It should coagulate or "thicken" the cream uniformly, giving it a clean, acid taste; and

d. The butter prepared from the ripened cream should possess an agreeable flavour and odour.

Pure culture starters are sent out either in a dry condition mixed with some inert powder, or in a liquid medium such as milk or bouillon.

They should be kept in a cool place and out of contact with contaminating dust, and when once opened the whole contents should be used immediately.

Since a tube does not contain enough bacteria to act as a starter for any considerable volume of cream, it is necessary for practical dairy purposes to build up a large bulk of milk containing enormous numbers of the organism. This is done by adding the pure culture to a gallon or two of pasteurized separated milk, and keeping it for about twenty-four hours at 65° to 75° F. until the whole is sour.

If more is needed, or where a continuous supply is

wanted, some of this soured milk may be added daily to a further amount of pasteurized milk. However pure they are at first, cultures kept going from day to day sooner or later become valueless; the organisms degenerate or slowly lose their vitality and ripening power, or the starter, in spite of careful precautions, becomes contaminated with objectionable organisms. This usually occurs in a week or two, after which time it is necessary to begin again with pure commercial or laboratory cultures.

Ex. 155.—Make a bacteriological analysis of any commercial starter you can obtain. If a powder, dissolve or distribute in sterile distilled water and plate out on litmus lactose gelatine and agar. Isolate and make pure cultures of each kind found. Examine and stain specimens and try the action of each on milk and other media. How many are liquefiers? Are any coliform organisms present? (Ex. 147). Are moulds abundant?

The chief object of the addition of a starter to cream is to immediately increase the number of organisms of a desirable kind to such an extent that they may suppress or overcome the activity of any bacteria of a deleterious, nature, which may have found their way into the cream. Usually from 8 to 10 volumes of starter are added to every 100 volumes of cream to be ripened.

Starters may be added—

(1) to cream raised by gravitation, which is necessarily several hours old and therefore contains considerable numbers of bacteria.

(2) to freshly separated cream, in which also a certain number of different kinds of bacteria are present, and

(3) to cream which has been subjected to the pasteurization process to get rid of all organisms in a growing state.

In the first case it is possible that the bacteria already there, may be of sufficiently vigorous stock to carry on the fermentations independently of the added starter. Such a state of affairs is likely to occur with comparatively old cream, and it is obvious that the addition of a starter in cases like this may have little or no appreciable effect: the operation is not worth the trouble it involves. The butter obtained where this practice prevails is not likely to be uniform at all seasons and may sometimes be of very bad flavour.

In the second case there is perhaps less danger of difficulty than where older cream is used, but irregular ripening may arise from organisms which have come from the farm and cowshed. The plan of adding the starter to pasteurized cream, while involving more expense, frees the starter from competition with adverse organisms and allows those of the chosen, desired type a free field for development. The keeping quality of the butter is improved as most of the organisms capable of giving rise to taints and bad flavours are destroyed by pasteurization. A more uniform grade of butter is produced and can be maintained with comparative certainty throughout the year. This method of using starters is adopted in Denmark and other countries where there is a progressive dairying industry, partly on account of the economic value of a uniform product and partly because the law requires the pasteurization of all milk which passes through creameries, with a view of assisting in the extermination of tuberculosis. The skim-milk and buttermilk which may ultimately be returned to the farms and consumed by susceptible young stock, is thus rendered free from the bacilli of the disease.

CHAPTER XXIII.

BUTTER.

1. WHEN cream is violently agitated in a churn or other vessel, the fat globules in it coalesce into larger or smaller grains or lumps which are removed from the separated serum or *buttermilk* and kneaded subsequently into an apparently compact mass of *butter*.

In this process it is as well to note that the fat globules do not as a whole lose their separate identity, but remain distinct and do not melt together as drops of oil do when brought into contact with each other.

Ex. 156.—Mount small portions of cream taken from a churn at various stages of the churning process up to the time when the butter "comes."

Press down the cover-slip.

Examine with a $\frac{2}{3}$ in. and $\frac{1}{6}$ in. objective and note the coalescence of the fat globules into small clusters.

Ex. 157.—Squeeze a very small piece of butter between the slide and cover-slip and examine with a $\frac{2}{3}$ in. and $\frac{1}{6}$ in. objective.

Is the butter homogeneous?

Make drawings of what you see.

It is practically impossible, by churning cream, to obtain the milk fats, pure and free from serum, so that butter always contains in addition to the fats a small but appreciable amount of protein, milk-sugar, and mineral substances as well as from 9 to 20 per cent. of water;

butter for sale in this country must not contain more than 16 per cent. of water.

The quantity of non-fatty material present varies considerably with the kind of cream used, the method of churning, and the amount of kneading or "working" to which the butter has been subjected, with the object of removing buttermilk. In most dairies a small quantity of salt is also added.

The following may be taken as the average composition of fresh and salt butter respectively :—

	Fresh Butter.	Salt Butter.
Water	14	12.5
Fat	83.5	84.5
Protein	.8	.5
Milk-sugar . . .	1.5	.6
Ash	.2	.1
Salt	...	1.8

Butter is composed of eight or more distinct kinds of fats, which are of course present also in normal milk. The chief of these are the fats *palmitin*, *stearin*, and *olein*, glycerides of palmitic, stearic, and oleic acids respectively, and *butyrin*, *caproin*, *caprylin*, *caprin*, and *myristin*, glycerides of butyric, caproic, caprylic, capric, and myristic acids.

The last five acids are soluble in water, and are known as volatile fatty acids, since they can be distilled over in a current of steam without change : the first three are insoluble and non-volatile.

Palmitin and stearin are solid white compounds, which occur also in beef and mutton fats ; olein is a liquid fat which only solidifies at or below about 40° F. ; it is found

in olive and cod liver oils. Butyrin, which is the characteristic fat of butter, melts at about 77° F.

The proportion of each of these in butter is subject to much variation, due to differences in the physiological condition of the cows and the food supplied to them. The amount of olein may rise to nearly half the total weight of butter fat. It is greatest in the early period of lactation, and may fall to about half the maximum at a later date. With a decrease in the size of the fat globules in the milk the olein decreases, and the butter becomes harder on account of the higher proportion of palmitin and stearin. Mangels, good grass, and green fodder generally tend to produce butter rich in volatile acids and of low melting point; hay and cotton-cake give harder butter, with higher melting-point.

Usually about 92 per cent. of the whole consists of palmitin, stearin, and olein, the rest being made up of the volatile fats, of which butyrin is the chief.

The approximate relative proportions are given in the following table:—

	Per Cent.
Palmitin, stearin, and myristin	49 to 52
Olein	37 „ 42
Butyrin	6 „ 7·5
Caproin, caprylin, etc.	2 „ 3

2. **Bacteria in Butter.**—The number and kinds of bacteria found in butter depend upon the bacterial contents of the cream from which it is made, the amount of added salt or preservative, and the age of the samples. In butter containing more than the usual amount of water, milk-sugar, protein, and other constituents of the cream, bacteria grow rapidly for a time, and 1 gram of a freshly-

made sample has been found to contain from 15 to 30 or 40 millions or more. The number, however, always decreases quickly on account of the want of aeration in the interior and the poor food-supply, for pure fats are not readily attacked by bacteria.

Jensen gives the following table, which indicates the decrease taking place in a marked sample of butter made from ripened cream, and also the difference in number of organisms on the surface and in the interior :—

	In Surface Layer.			In the Interior.		
	Total.	Liquefying Organisms.	Oospora Lactis.	Total.	Liquefying Organisms.	Oospora Lactis.
Fresh sample	12,000,000	20,000	80,000	12,000,000	20,000	80,000
1 week old	14,000,000	20,000	580,000	2,000,000	12,000	20,000
2 weeks old	16,000,000	...	200,000	2,000,000	...	400
4 ,, ,,	2,700,000	0	480,000	360,000	0	0

The numbers which have been found in butter are many and of various species. Some of them may be considered as accidental impurities introduced from the air or water-supply of the farm and dairy ; others are more or less characteristic of most samples of butter, and come into it from the milk or cream out of which it is manufactured. Among the organisms usually present the following may be mentioned :—

(*a*) Lactic acid organisms, especially *Str. lacticus* and its allies.

(*b*) *Bs. lactis aerogenes.*

(*c*) *Bs. fluorescens liquefaciens.*
(*d*) *Micrococcus acidi lactici* (Krüger).
(*e*) *Oospora lactis.*
(*f*) Lactose-fermenting yeasts.
(*g*) Certain fungi, especially species of *Mucor*, *Penicillium*, and *Cladosporium.*

Less frequent, but yet commonly found are :—

(*a*) *Sarcina lutea.*
(*b*) *Bs. vulgatus.*
(*c*) Species of *Proteus.*
(*d*) True *Bs. coli.*
(*e*) Species of *Streptothrix.*

3. **Flavour and Aroma of Butter.**—In the manufacture of butter great attention is paid to the development of an agreeable flavour and pleasant aroma, since it is upon these two qualities that the commercial value of the product largely depends.

Flavour, which refers to taste, and aroma, which is another term for smell or odour, being usually perceived together when butter is eaten, are often confused.

They are, however, distinct qualities, and not necessarily connected with each other, for butter may possess an agreeable aroma with an objectionable flavour, and vice versa ; nevertheless the same substance may influence both these qualities sometimes.

What the compounds are which give to butter its characteristic odour and taste are not known. The evidence hitherto obtained upon the matter points to the conclusion that some of them are sweet-smelling esters derived from the fermentation of milk-sugar, while others are decomposition products of the casein of the butter. Moreover, it is to the mixture of these that aroma and

flavour must be attributed, and not to any one substance only.

Much research has been carried on to determine the kind of organisms which produce perfect flavour and aroma. No single species of bacterium, however, has been found to be able to give the desired result when used as a pure culture and inoculated into sterilized, pasteurized, or ordinary cream. Some of them isolated by Conn, Storch, Weigmann, and others, have given a good butter when used singly in this way, but not one with all the good qualities of a first-class sample.

It is pretty well established that the organisms which influence or induce flavour and aroma do not belong to the class which are specially concerned with the lactic acid fermentations, but rather to those which induce alkaline and putrefactive changes.

On this assumption it is likely that they would ruin the butter if allowed to get the upper hand or a free field for development; they are, however, ordinarily kept in check by the lactic acid bacteria.

4. **Taints and Defects of Butter.**—Many of the faults which reduce the flavour and market-value of butter are due to want of skill in churning and general dairy management, as well as to the nature of the food consumed by the cows.

The fats of milk may be of low melting-point, and the butter consequently soft and oily, owing to the use of linseed-cake as food, or the butter may be too hard from the consumption of too much cotton-cake by the cows. Streakiness, or mottling, may be caused by irregular salting or the presence of specks of casein owing to over-ripening of the cream. Offensive flavours may arise from giving the cows turnips or cabbages, or

from their eating garlic and other weeds of the meadows and pastures.

Bad odours of the cowshed and dairy are also readily absorbed by milk, cream, and butter.

With these faults we are not here specially concerned. There are, however, many troubles of the buttermaker due to the action of bacteria, and some of these require notice; those of comparatively common occurrence are rancidity, tallowy flavour, turnip flavour, bitterness, fishy taste and smell, and mustiness or mouldy odour.

(i) **Rancid Butter.**—There is no chemical change of butter which has been so much investigated, or which has given rise to so much controversy as that which results in the condition usually spoken of as *rancid*. A large number of factors are known to be concerned in the production of rancid butter, but the exact influence of each is still a matter of doubt.

It is difficult to define "rancidity," and many of the conflicting results of investigation have been connected with the want of exact knowledge of what is meant by the term "rancid."

Butter, especially if not skilfully made, when kept for a long time loses its pleasant aroma, and gradually takes on a pungent or acrid smell and taste, which, though easily recognized, is difficult to describe. Simultaneously with the development of the rancid flavour the butter generally becomes more or less acid from the production of free fatty acids within it. If left exposed to light, the yellow colour disappears from the surface layers, which become transparent at the same time; little by little these changes penetrate to the centre of the mass, the progression reminding one of the ripening of a soft cheese.

In rancid butter the following substances are usually found, few or none of which are present in fresh butter :—

1. Free fatty acids ;
2. Certain oxy-acids ;
3. Aldehydes and ketones ;
4. Esters or ethereal salts, especially of butyric and volatile fatty acids.

The amount of each varies considerably with the age of the sample and the extent of the action of the various agents to which these products are due.

Duclaux and several of the earlier investigators attributed the peculiar rancid taste and odour to free butyric acid or some allied fatty acid, but that is not the case.

Very frequently it is found that high acid content goes along with strong rancid conditions ; but stored butter may become strongly acid and possess an objectionable flavour sufficient to render it uneatable without being rancid ; on the other hand, rancid butter may contain little or no free acid.

The free fatty acids arise through the splitting of the fats, glycerine being set free at the same time. Some of the glycerine undergoes oxidation, and aldehydes and ketones with rank taste and evil smell are produced.

Moreover, oxygen is absorbed by unsaturated compounds, such as oleic acid.

None of these compounds, however, give the strictly rancid flavour.

According to Amthor and Reinmann and others, the characteristic rancid odour and flavour is that of the esters of the volatile acids, and especially of ethyl-butyrate, the ethyl ester of butyric acid.

Amthor believed that the ethyl group of the butyric

ester was derived from milk-sugar by fermentation; but Jensen suggests that it more likely comes from an intermediate product, monobutyrin, obtained in the decomposition of tributyrin.

The conditions which govern and accelerate rancidity are: access of air, light, warmth, and moisture, high casein and milk-sugar content, and the presence of various micro-organisms.

The splitting of the fats apparently arises in part through the action of moisture, carbon dioxide, and lactic and other acids produced by fermentation of the milk-sugar, and also to a considerable extent through the agency of micro-organisms, especially *Bs. fluorescens liquefaciens*, *Oospora lactis*, as well as various yeasts and "mould" fungi. *Penicillium glaucum* and certain species of *Aspergillus* are able to hydrolyse fats by means of lipase, an enzyme which they possess.

Free butyric acid arises through the decomposition of butyrin in this way, and not as the result of butyric fermentation: the latter is the work of anaerobic species of bacteria (p. 285) which play very little or no part in the development of rancidity in butter.

The oxidation of oleic acid and the production of aldehydes and ketones is to a certain extent due to the direct chemical action of the air under the influence of light, and the butter may develop a tallow-like, but not rancid, character and taste by this means alone. Butter does not become rancid when oxygen is completely excluded. However, sterilized butter does not develop the rancid flavour and odour even when exposed to light and air; so that the action of air would appear to be chiefly important because of its assisting the growth of bacteria and fungi of the aerobic class.

The recent researches of Reinmann and Jensen have shown that rancidity is mainly the work of aerobic bacteria and fungi, conditioned by the presence of air, moisture and the other factors already mentioned. Its full development is associated with a number of different chemical reactions which occur among the decomposition products of the fats, caseinogen, and milk-sugar; the changes are not quite so complex, perhaps, as those which take place in a ripening cheese, but are in many respects analogous to the latter.

There is much evidence to support the view that micro-organisms are chiefly responsible for the initiation and maintenance of the changes involved. A sample of normal butter containing a considerable amount of casein, sugar, and other materials upon which bacteria thrive becomes rancid much more rapidly than a specimen containing a smaller quantity of these compounds.

Butter made from sterilized cream keeps fresh for a long time, in spite of the fact that it contains an abundant supply of food for bacteria; however, when inoculated with a small piece of rancid butter, the whole becomes rancid very quickly.

Antiseptics, such as salicylic acid and formalin, check it, and keeping the butter in a cool place tends to prevent it. That the change is not entirely due to enzymes is deduced from the observations that antiseptic agents which allow these to act, but check the chemical activity of living organisms, stop the development of rancidity.

No single species of bacterium or fungus which has been isolated from rancid butter has been found capable of inducing the rancid taste and flavour in a sterile sample. The production of this objectionable taint is the result of the interaction of several kinds of organisms

of which the following appear to be the most important, since they are practically always met with in abundance in rancid butter :—

1. One or two forms of lactic acid bacteria ;
2. *Oospora lactis* ;
3. Yeasts, some of which form rose or yellow colours on gelatine and agar ;
4. *Cladosporium butyri*, a species of hyphomycete belonging to a widely distributed genus.

Less frequently certain "moulds" (species of *Mucor* and *Penicillium*) are present, as well as *Bs. lactis aerogenes* and *Bs. fl. liquefaciens.*

(ii) **The Tallowy Flavour** and appearance may be brought about by the decomposition of the fats and the production of free fatty acids. The cause of the change, as already pointed out, is sometimes bacterial ; but in other cases the tallowy taste and texture is the result of purely chemical processes, namely oxidation and hydrolysis under the influence of light and moisture, especially accompanied by somewhat high temperature. Storage of the butter in a dark, cool, and comparatively dry place will usually check this trouble.

(iii) **Turnip Flavour in Butter.**—When large quantities of turnips are used for the feeding of cows the milk becomes tainted, and the cream and butter obtained from it possesses an objectionable flavour and odour of these roots.

The exact cause of the turnipy flavour is not yet understood perfectly. In some cases the milk as it is drawn from the cow possesses the peculiar flavour or may absorb it from the air of the cowshed where turnips are stored, or used for food ; in most instances, however, the trouble arises later and is a bacteriological one.

Weigmann and his pupils have shown that the dung of cows fed with large amounts of turnips contain certain varieties of *Pseudomonas* belonging to the *coli*-group of bacteria which are able to give rise to the taste and odour of turnips in varying degree when introduced into milk, cream, and butter. Presumably these gain access to the milk in particles of dung, for when the udders of the cows feeding on turnips are brushed and washed and the dung disinfected with lime the milk and the cream and butter derived from it do not develop the turnipy taste. These organisms produce the strongest development of the objectionable taste when working in combination with varieties of *Streptothrix* and certain fungi, especially a form of *Penicillium brevicaule.*

Foul odours may arise in butter which is contaminated with dirty water, dung, and other materials containing *Bact. mycoides*, *Bact. vulgatus*, *Bs. fluorescens liquefaciens*, and other organisms of the putrefactive group.

Bitterness may be due to food of the cow or the activity of bacteria.

Fishy and cheesy odours and tastes have, in certain instances, been traced to the work of moulds and species of *Torula* (p. 394).

5. **Salt in Butter.**—The addition of salt has a very beneficial influence upon the keeping quality of butter and in small amounts assists in preserving it free from taints.

The number of organisms present in salted butter increases much more slowly, and the ultimate decrease which occurs begins at an earlier period than in unsalted samples.

This is evident from the following table given by Fettick :—

	Number of Organisms in	
	1 gram of unsalted butter.	1 gram of same butter with 3 per cent. of salt added.
Fresh	29,303	25,408
10 days old	72,782	23,539
20 " "	400,178	242,588
70 " "	559,771	327,938
98 " "	643,040	302,430

Fettick also showed that the moulds of butter, of which species of *Penicillium*, *Oospora*, and *Mucor* are most prevalent, are very rapidly reduced in salted butter, but continue to develop for a considerable time when the material is left unsalted.

	Number of Moulds in	
	1 gram of unsalted butter.	1 gram of same butter with 3 per cent. of salt added.
Fresh	687	784
10 days old	9,375	750
20 " "	99,125	241
70 " "	263,429	0

This is an important result, since some of the objectionable oily, rancid, fishy, and cheesy flavours of butter have been traced to this class of organisms. More than 3 per cent. of salt would appear to be disadvantageous, since larger amounts inhibit the lactic acid bacteria without interfering much with the growth of *Bact. coli*, *Bact. l. aerogenes*, *Bs. subtilis*, and *Bact. putrificus*. Highly salted butter is liable to develop taints which do not appear in samples containing less of this preservative, a point which has to be borne in mind by the producer of butter for the export trade.

CHAPTER XXIV.

CHEESE.

1. **Rennet.**—For the coagulation of the caseinogen of milk in the manufacture of cheese the salted and dried inner membrane of the so-called "fourth" or true digestive stomach of very young calves is frequently employed under the name of *rennet.*

Such material contains a *coagulase* or coagulating enzyme, termed *rennin* or *chymosin*, and also a certain amount of pepsin, the former precipitating paracasein in milk, while the latter in acid solutions breaks down the curd into simpler proteins.

Rennin is found in the stomachs of most young mammals, and commercial extracts are prepared from those of dogs and pigs, as well as calves.

A similar coagulase is present in certain seeds, in the leaves and stems of Bedstraw (*Galium verum*, L.), and in the flowers of the Artichoke (*Cynara Scolymus*, L.). Fitz and Hueppe showed that it is possessed by *Bacillus butyricus*; Warrington found it in *Bs. fluorescens liquefaciens*, and Vignol proved its existence in *Bs. vulgatus.* Certain varieties of *Proteus*, *Bs. prodigiosus*, and some of the lactic acid organisms have the power of coagulating caseinogen and subsequently dissolving it by means of a pepsin which they secrete, the former action being most in evidence in cultures kept at 20 to 25° C., while their peptonizing property is most active at 37° C.

Sometimes small pieces of the dried and salted stomachs are added to milk in order to coagulate it but most cheesemakers now use a brine extract of rennet containing the active principle—rennin. Home-made extracts are prepared by soaking the "vells" or stomachs in .3 to .6 per cent. solution of salt for three or four weeks, after which the liquid is filtered through charcoal. The extract should be dark-coloured but clear, turbidity indicating objectionable decomposition.

Commercial extracts in much stronger brine (16 per cent. solutions) are available as well as rennet "powders" and "tabloids": these are more reliable, being more regular in strength than home-prepared material, and less likely to contain bacteria and other impurities which give rise to tainted or faulty cheese.

Rennet is a very active substance, exceedingly small amounts being able to coagulate large quantities of milk. One part of ordinary extract, which is itself a very weak solution of the active enzyme, will very frequently curdle as much as 5000 to 6000 times its volume of milk; it has been calculated that one part of pure rennin will coagulate 30 million parts of milk.

The extracts are very sensitive to the action of light, heat, and certain chemical agents. They should be kept cool and in the dark, not in glass bottles, for the alkali of the glass dissolves out and damages the enzyme.

Rennin is most active in neutral or in very dilute acid or alkaline solutions; strong alkalis or strong acids soon destroy it.

Its coagulating power increases from about 10° C., below which it ceases to act, up to the optimum, about 39 to 42° C.; above this temperature it decreases until at about 70° C. its strength rapidly diminishes even in

strong solutions which are much less sensitive than dilute extracts.

Certain antiseptics, especially borax and formalin, damage its curdling power, but chloroform, thymol (.1 per cent.), or carbolic acid (.5 to 1 per cent.) have little effect upon it, although they check the activity of bacteria.

In the stomachs of the young animals, from which home-made rennet extracts are sometimes prepared, several different species of bacteria are often found. According to Thöni such bacterial flora consists of—

(i) A group of organisms, such as *Bs. subtilis*, *Bs. vulgatus*, species of *Sarcina* and *Streptothrix*, which have little effect on the quality of the extract and soon disappear;

(ii) Some of the useful lactic acid organisms;

(iii) *Bs. coli* and *Bact. lactis aerogenes,* which may multiply and ultimately contaminate the curd; this group is the most to be feared by the cheesemaker.

Ex. 158.—Repeat Exs. 113, 114.

Ex. 159.—Make a bacteriological analysis of home-made and commercial rennet extracts on the lines indicated in Exs. 125, 147, 155.

2. **Cheese.** (i) **Composition and ripening.**—When rennet is added to milk which has been allowed to become slightly sour, a solid curd or coagulum is thrown down, and a greenish yellow liquid, the whey, is separated. This curd is the raw material from which various kinds of cheese are made. It consists chiefly of a protein substance termed casein (sometimes paracasein, see p. 237), along with a considerable proportion of the fat, sugar, and mineral matter originally present in the

milk. About one-half of the solids in the milk is recovered in the cheese. The fresh curd is manipulated in a great variety of ways, and from it a correspondingly large number of kinds of cheese are made, differing in size, form, texture, and other physical characters. The different kinds vary considerably in chemical composition. The firm or hard cheeses obtained by coagulation at temperatures between 30 to 35° C. contain from—

28	to	35	per cent. of	water,
25	„	35	„	fat,
23	„	35	„	proteins,
1	„	3	„	sugar, lactic acid, and
2.7	„	4	„	ash.

The soft cheeses prepared from curd precipitated at temperatures below 30° C. contain from—

45	to	50	per cent. of	water,
20	„	30	„	fat,
18	„	20	„	proteins,

and small amounts of sugar, lactic acid, and mineral matter.

Freshly made cheese is at first of a firm, elastic texture, is slightly acid, practically insoluble in water, indigestible, and possesses no very definite cheesy odour or taste. Sooner or later, however, very extensive physical and chemical changes occur within it, and the cheese is said to ripen. Much of the insoluble material becomes soluble and is then easily digested, at the same time the different varieties of cheeses develop their own peculiar characteristic aroma and flavour. The chemical changes which take place during the ripening process are very complex, and, in spite of much investigation,

are not very clearly understood. The milk-sugar which can be detected in freshly made cheese generally disappears in a few days, and a watery extract of a ripe one contains none of it. The fat undergoes very little alteration. Various acids which are not found in the fresh cheese appear in the ripened product. Lactic acid and its salts are met with, and small amounts of acetic, formic, propionic, butyric, caproic, valeric, and other acids are frequently present either in the free state or united with ammonia or other bases. Ripe cheese always contains a certain amount of free non-volatile fatty acids, such as oleic, palmitic, and stearic acids. The most important changes occurring during the ripening process are those which are concerned with the cleavage or breaking down of the casein. This protein undergoes gradual hydrolysis as ripening proceeds, simpler compounds arising at each stage of the process. At first the casein is changed into caseogluten, a substance closely resembling casein, but differing from it in being soluble in a solution of salt. Later the more soluble and non-crystalline proteoses (caseoses) are formed; from these less complex peptones (caseones) are produced, and later the peptones give rise to the simpler soluble crystalline amino-acids, the chief of which are glycocoll, alanine, leucine, tyrosine, phenylalanine, and tryptophane. From the amino-acids originate small amounts of various amines, such as putrescine and cadaverine, to which may be attributed some of the objectionable flavours and odours of old cheese. Eventually ammonia is formed, and in some instances may be set free in very small amounts, as in the soft cheeses, but it is more frequently present in combination with various acid compounds.

Van Slyke and Hart found in Cheddar cheese twenty-four hours old that only about 6 per cent. of the total nitrogen in it was soluble in water, whereas in a ripened one, nine months old, 53 per cent. was soluble. The changes in the proteins during the ripening of cheese resemble those observed in peptic or tryptic digestion of proteins, and, as far as they go, are similar to a process of putrefaction, proteoses, peptones, and amino-acids being produced in both cases. In ordinary putrefaction, however, certain compounds are produced, especially oxyacids, phenol, indole, skatole, and various gaseous substances which are never met with in normally ripened cheese. Possibly the milk-sugar, or the organic phosphorus compounds have an inhibiting effect on the putrefactive bacteria, or the organisms which utilize carbohydrates check or destroy them.

The changes in the constituents of cheese, which have just been described, take place slowly, and almost uniformly, throughout the whole mass of hard pressed varieties, such as Cheddar, Emmenthal, and Edam, the time taken to obtain a thoroughly ripe condition being several months. Most of the whey is removed from the curd at the time of manufacture of this type of cheese. On the other hand, in the soft cheeses a much greater amount of whey remains in the curd, and ripening takes place at a rapid rate, the casein being broken down into soluble compounds in a few days. Ripening begins on the outside and proceeds inwards, a section of a partially ripened specimen showing a distinct outer layer of soft, ripened, creamy material, surrounding an interior of firm, white, unaltered curd.

(ii) **Aroma and flavour.**—During the ripening process

cheeses develop a characteristic aroma or odour, which is well-marked and quite distinct from that of fresh curd. The pleasant, sourish smell of a ripening cheese is probably due, to some extent, to minute traces of formic, acetic, and similar free volatile fatty acids present in it. Propionic and butyric acids, which are generally found in greater amount, have a penetrating and somewhat rancid smell. Isovaleric acid has a characteristic old-cheesy odour, and the unpleasant smell of the rind of certain overripened cheeses has been traced to the presence of caproic and caprylic acids. The peculiar odours of cheeses are not entirely due to free, fatty acids ; nitrogenous compounds, such as amines and ammonia, contribute their share. Putrescine and cadaverine, both of which have a sharp, objectionable odour, have also been isolated from cheese. The flavour or taste of a good nutty cheese appears to be connected with the amino-acids, most of which have a mild, sweetish taste. According to Jensen, the delicate, nut-like flavour of the best Emmenthal cheese increases with an increase in the amount of those soluble nitrogenous compounds which cannot be precipitated from their solutions by phosphotungstic acid, a class which includes the amino-acids. Many of the more complex protein substances have each a characteristic taste and smell, but nothing definite is known about their influence in the production of good flavour and aroma in ripe cheese. In some instances considerable amounts of bitter substances are produced, but little is known of their nature or origin : they only arise in cheeses undergoing putrefaction or abnormal decomposition, or where little or no salt has been used in the manufacture. Many of the peptones have a bitter taste, but in normally ripened cheese they

are never at any time abundant, and their bitter flavour appears to be removed by common salt in a manner not yet understood.

(iii) **Causes of the ripening of Cheese.**—While the general facts of the ripening process are fairly clear, the causes which operate in the production of the chemical and physical changes involved in the process are still imperfectly understood, and much controversy has arisen in regard to them. It is, however, agreed on all sides that ripening is due chiefly, if not altogether to the activity of bacteria and fungi. The process only goes on when these are present, and cheese prepared from sterilized milk, or kept under conditions which destroy living organisms, do not ripen. Russell and Babcock state that they found cheese would ripen when immersed in chloroform, and suggested that the process was due to the action of galactase, a trypic enzyme normally present in milk and capable of hydrolysing casein. It is very doubtful, however, if the action of bacteria was precluded in their experiments, and careful investigations by other workers do not support Russell and Babcock's view. It is probable that galactase plays some part in the breaking down of proteins in cheese, but its influence is small. Another proteolytic enzyme, namely pepsin, is introduced into cheese in the rennet used for curdling the milk, and this no doubt also takes a share in the ripening process. An increased addition of rennet is followed by an increment in the amount of those soluble nitrogenous compounds which are characteristic of peptic digestion. Moreover, pure pepsin added to cheese hastens the ripening process, more particularly in Cheddar and similar varieties, where considerable acidity is developed in the early stages of

manufacture. The ripening process appears to be a peptic proteolysis at first; later, when less acid or more alkaline conditions prevail, it resembles tryptic digestion. Although galactase and the pepsin of rennet are probably concerned in some degree with the ripening of cheese, it is to bacteria that the chief cause must be attributed. Various kinds of organisms are present in the milk as it comes from the teats of the cow, or find their way into it from the air and utensils of the stable and dairy. With the rennet large numbers are added, since each c.c. of the extract ordinarily used contains from 25 to 35 millions of bacteria.

Many elaborate investigations have been undertaken to ascertain the number and kind of bacteria present in cheeses at various stages in the ripening process. It is usually found that in the fresh curd there are fewer bacteria than in the slightly acid milk from which the curd has been precipitated by rennet, large numbers being of course removed in the whey. According to Russell and Weinzirl, in a new Cheddar cheese there is a decrease in the bacterial content during the first twenty-four to forty-eight hours, due apparently to a reduced temperature and to a further loss of whey in the press. From this point a rapid rise in numbers takes place until a maximum is reached, when a gram of cheese will contain from 90 to 150 millions or more of bacteria. The time taken for the development of the maximum number of organisms depends upon the kind of cheese, the temperature at which it is kept, and other factors; in Cheddar it usually occurs between the tenth and twentieth day. A steady decline in numbers now goes on for several weeks or months until the cheese contains comparatively few living organisms. By microscopic examination of

sections of cheese the bacteria in it can be seen *in situ* and their distribution, development and form at various stages in the ripening can be studied. Heaps or colonies of larger or smaller extent are found distributed irregularly throughout the mass, a fact which makes it difficult to obtain concordant quantitative results regarding the number of bacteria present in cheese. The colonies are generally pure, but occasionally two or more kinds may be intimately mixed. In the spaces between these single organisms, small groups or isolated spores are generally seen, but in some cheeses, especially those which have undergone ripening for some time, the spaces are free from living bacteria.

A very large number of species of bacteria have been isolated from different varieties of cheese. Many of them have been imperfectly described, and a list cannot be given here. They may, however, be classified for convenience into the following groups:—

(i) Organisms which are capable of fermenting milk-sugar with the production of lactic acid without gas formation, *e.g. Streptococcus lacticus*, Kruse.

(ii) Those which give rise to lactic acid and considerable amounts of free carbon dioxide and hydrogen, *e.g. Bs. acidi lactici*, Hüppe, and *Bact. lactis aerogenes*, Esch.

(iii) Proteolytic bacteria, which digest or decompose casein.

(iv) A group of miscellaneous kinds which appear to be without any power of attacking the carbohydrate or proteins in cheese, and which die out or remain dormant in it.

Much of the controversy which has arisen in regard

to the ripening process has been concerned with a determination of the function or part played by these various groups of bacteria, and how the several kinds affect the different components of cheese. The chief views held by the different workers at the problem are discussed below.

(*a*) Duclaux, who was the first to make a systematic study of the ripening process, isolated from Cantal cheese ten species of bacteria, the most important of which were placed by him in the genus *Tyrothrix*. The latter were all somewhat large bacilli, which grew readily in milk and other liquid media into large threads sometimes 100 μ to 120 μ long, and formed spores which were able to resist a temperature of 100° C. for a short time. They are now known to be very similar in many of their characters to the hay bacillus (*Bs. subtilis*) and the potato bacillus (*Bs. vulgatus*). When introduced into milk Duclaux found they were able to decompose the casein with the production of an albumose, amino-acids, and other compounds met with in ripened cheese, and concluded that they were the chief agents in the ripening process. The power of these organisms to peptonize casein Duclaux considered was due to the secretion by them of a proteolytic enzyme, *casease*, which diffused slowly throughout the substance of the cheese. Since the peptonizing bacteria are checked by the presence of much acid, they only begin their specific work after the initial lactic fermentation is over, and the acid produced has been destroyed or neutralized. This view has been supported by Adametz, Von Klecki, Winkler and others, and a pure culture of *Bacillus nobilis*, an aerobic form of a *Tyrothrix* obtained from Emmenthal cheese, has been sold for cheesemaking under the name "*tyrogen*." Its use, however, has not met with success. If the peptonizing

group of bacteria such as species of *Tyrothrix* and allied spore-producing kinds were the chief cause of ripening, it would be expected that they would be more abundant in a ripened cheese than in one which has undergone little change, but this is not the case. They are never abundant in cheese at any time, and are generally absent altogether in Cheddar and other hard cheeses a few months old : even when added in large numbers to the curd they fail to increase, and die out usually before any sign of ripening can be detected.

(*b*) The view that the lactic bacteria are the chief agents in the ripening of cheese was strongly supported by Freudenreich, and many of his pupils still maintain this opinion. The common lactic bacteria are by far the most abundant organisms met with in cheese of all kinds, and in the early stages of the ripening comprise more than 95 per cent. of the total bacteria present. Weinzirl found that of the total bacteria present in Cheddar cheese about 74 per cent. were lactic forms near *Streptococcus lacticus* and 22 per cent. allied to *Bs. acidi lactici* (Hüppe), leaving only 3 or 4 per cent. for all the other species, and similar results have been obtained by Percival and Mason in Stilton cheeses. The lactic bacteria uphold their supremacy almost to the end of the ripening period, and in practice it is found that cheeses which contain large numbers of these organisms are much superior in quality to those in which fewer are found. Cheese made from milk from which they are absent always ripens slowly and abnormally, whereas when a culture or "starter" of lactic acid bacteria is added such milk will produce a good cheese.

In hard cheeses, where the organisms other than lactic bacteria are in the early stages in greater pro-

portion than 3 or 4 : 96 or 97, the ripening is likely to be unsatisfactory and result in the production of a cheese of poor flavour and aroma.

Freudenreich isolated from Emmenthal cheese several cocci and rod-shaped bacteria which produced lactic acid and curdled milk, and at the same time broke down the casein into soluble proteins when the acid was neutralized with chalk. Gorini states that some bacteria act as lactic acid producers at higher temperatures, and peptonize casein under the cooler conditions of the cheese store-rooms.

(*c*) Weigmann and others consider that the ripening of cheese is a much more complicated problem than that which is suggested by Duclaux and Freudenreich. Many workers hold that the specific lactic organisms do not ripen cheese. It is impossible under ordinary conditions of manufacture to avoid the access of these bacteria to the milk and fresh curd, and they find the latter a suitable medium in which to grow and multiply extensively, but their influence upon the ripening is indirect only. It is suggested that they destroy or check some kind of bacteria which are detrimental to the normal development of taste and flavour, and produce an acid condition which is essential to the growth of the peptonizing group and the action of their enzymes. In some instances sour whey or other acid starter is added during the manufacture of the cheese in order to ensure that the ripening shall proceed in a normal manner. Ripening is assumed to be a kind of symbiotic fermentation in which the lactic organisms use up the oxygen and prepare the ground for the anaerobic species which are supposed to be the bacteria chiefly responsible for the degradation of the proteins in the cheese. Weigmann

obtained two species of anaerobic bacteria, *Paraplectrum fœtidum* and *Clostridium licheniforme*, from cheese; they belong to a rod-shaped, spore-bearing class which produce butyric acid, and are supposed to be concerned with the ripening of the cheese and the development of aroma and flavour in it.

(*d*) Boekhout and De Vries are inclined to believe that, in the case of Edam cheese at any rate, the cause of ripening is not to be found in the work of bacteria during or after the lactic fermentation has taken place, but is due to the enzymes produced by peptonizing bacteria which get into the milk in the stable and in the dairy. These organisms begin their work when the cheese is made, although their activity is soon checked by the development of the lactic bacteria. However, the proteolytic enzymes already produced by them are not destroyed, and these substances continue to break down the paracasein and ripen the cheese.

(*e*) The author is of opinion that the lactic acid organisms are chiefly responsible, in an indirect manner, for the ripening of most cheeses, the proteolytic enzyme upon which the process depends being apparently intracellular, only coming into operation at or about the time of death of the bacteria.

The organisms, of which many millions per gram can be detected in unripe cheese, disappear as ripening proceeds, their bodies being broken down under the influence of their intracellular trypic enzyme; after this autolytic digestion the enzyme set free from the dead bacteria continues its proteolytic action upon the surrounding curd.

Ex. 160.—Take a small piece of cheese, smooth one side with

a razor, and press the smooth surface gently down on a clean cover-slip, so as to leave an impression of the cheese upon it.

Pass the slip through the flame and dip it in ether for two minutes and then in absolute alcohol for one minute to remove the fat. Stain for ten minutes with Nicolle's carbol-thionin solution (saturated solution of thionin in 90 per cent. alcohol, 5 c.c.; 1 per cent. solution of carbolic acid, 50 c.c.). Examine with low and high powers.

Note the forms of the bacteria present. Are they cocci or rods?

Are the organisms arranged in heaps or colonies, or distributed singly?

Ex. 161.—Try the above experiment with cheeses of different ages. Make observations on the form and arrangement of the bacteria in fresh, half-ripe, and fully ripe cheeses.

(iv) **Ripening of Soft Cheeses.**—The chief differences between the hard and soft varieties of cheese have been incidentally mentioned earlier in the chapter. To the soft kinds no artificial pressure is applied, the whey in the curd being merely allowed to drain away by gravitation. Such cheeses, of which Camembert may be taken as one of the best known examples, frequently contains from 45 to 50 per cent. of water. On account of this high moisture content, bacteria grow in them very rapidly, and moulds soon cover their external surfaces.

The chemical changes go on quickly, progressing from the outside inwards, a partially ripened specimen showing a firm core of acid curd, around which is a layer of buttery, semi-liquid or digested casein of variable thickness.

The casein is broken down into proteoses, peptones, and amino-acids: the decompositions are similar in kind to those found in the ripening of hard cheeses, but they

are carried further and at a more rapid rate, complete ripening occupying not more than three or four weeks, instead of as many months.

In normal examples tryptophane, indole, skatole, sulphuretted hydrogen, and other products of putrefactive fermentations are absent.

Good Camembert cheese contains about .25 per cent. of ammonia when ripe, mainly combined with acid radicles ; only in over-ripe samples can free ammonia be detected, its occurrence in considerable amounts indicating putrefaction and decay.

The biological factors in the ripening of soft cheeses are different from those of the hard varieties ; in the latter, bacteria alone are mainly responsible for the chemical changes, while the former are ripened chiefly by means of fungi.

In both types, of course, the lactic acid organisms are abundant, and assist in the preliminary changes occurring in the cheeses ; but in the soft Camembert cheese *Penicillium candidum*, Rogers (by some authors named *Penicillium Camembertii*) is the active ripening agent, the mycelium of this fungus secreting a proteolytic enzyme which diffuses into and peptonizes the acid curd.

Always associated with it is *Oospora lactis* and various yeasts, to which the pleasing aroma and flavour are in part due, for cheeses ripened with pure cultures of *P. Camembertii* do not develop the characteristic Camembert taste.

For a successful result an abundant development of *Streptococcus lacticus* and allied lactic organisms is essential, otherwise foul-smelling decompositions are certain to arise.

In three or four days the two fungi just mentioned

appear as a white felt, covering the surface of the cheese. The acidity decreases and an alkaline condition is established in the rind and a thin layer immediately beneath it, softening and digestion of the curd occurring at the same time.

An excessive growth of *Oospora lactis* must be avoided, as this leads to the production of an objectionable flavour.

3. Intermediate between the hard and soft cheeses are Stilton, Gorgonzola, and other varieties which are drier, firmer, and much larger than the soft forms, but not so dense as the Cheddar and hard types. Upon the rind of these kinds moulds and yeasts grow abundantly, and after the lactic acid organisms have reached their maximum development and begun to disappear, the whole body of the loosely-packed curd becomes penetrated by the mycelium of a *Penicillium*. In the fissures in the interior of the cheese the mould develops its characteristic blue-green conidiophores, a well-ripened Stilton showing, when cut across, a series of branching blueish veins.

4. **Some cheese defects.** (i) **Gassy or blown cheese.**—One of the most frequent and most troublesome difficulties with which the cheesemaker has to contend is the production of "gassy" or "blown" cheeses, which invariably possess a disagreeable flavour. Throughout the substance of the cheeses large numbers of more or less spherical cavities or "bubbles" are formed. In some cases the holes are not larger than a pin's head, while in others they may grow to a large size, and give rise to cheeses which are cracked, swollen, and distorted in shape.

The holes or bubble-like cavities are produced by "gassy" fermentation of the milk-sugar, and develop

to the greatest and most serious extent in cheeses of the Emmenthal and Gruyere types, which are manufactured from somewhat sweetish curd.

Sometimes the holes appear in the early stages of manufacture, the curd in such instances containing enough gas to make it float in the whey. Frequently, however, the trouble only shows itself when the cheese is in the press or in the ripening room.

The organisms which are responsible for the majority of "gassy" cheeses are varieties of *Bact. coli* or *Bact. lactis aerogenes*, especially those which give rise to much gas when grown in milk-sugar media.

Bact. Schafferi and *Bact. Guillebeau* of Freudenreich belong to this class, and other organisms closely allied to *Bact. lactis aerogenes* have been isolated and shown to be the cause of "blown" cheese and bubbly curd by Peter, Schneebeli and others.

Bolley and Hall found a close relationship between this trouble and contamination of the milk with cow-dung, and this appears to be specially true when the latter comes from animals suffering from digestive disturbances, brought on by the consumption of wet grass, clover, and other succulent green foods.

There is some evidence that some of the organisms associated with mastitis or inflamed udder sometimes have a causal connection with spongy curd. *Bs. Guillebeau*, Freud., and *Micrococcus Sornthalii*, Adametz, are said to possess this double character.

Certain varieties of the butyric acid organisms and yeasts probably give rise to gassy cheeses also. Various methods are adopted to get rid of gassy fermentation in cheese.

Every effort should be made to reduce contamination

with animal fæces; cleanliness at the farm will go a long way towards preventing it.

The lactic acid fermentation inhibits the organisms which cause it, and the addition of sour milk "lange wei," or a specially clean starter, is advisable where sponginess of curd is expected.

Ripening at low temperatures is also useful. The addition of .1 per cent of potassium nitrate (saltpetre) to the milk for cheesemaking is beneficial. Boekhout and De Vries have shown that the gas-producing bacteria break up the milk-sugar in the milk or cheese with the evolution of carbon dioxide and hydrogen gases, but when potassium nitrate is present they obtain the oxygen which they need from this compound, leaving the milk-sugar to be changed by the ordinary lactic acid organisms.

Ex. 162.—Make a fermentation or Wisconsin curd-test with clean milk, and one to which a small amount of cow-dung or pure culture of *Bact. lactis aerogenes* has been added.

Take two glass jars or tin vessels each holding about a pint; they must have tin or glass covers or lids. Sterilize them in the hot-air sterilizer; when cool fill the vessels about two-thirds full of the milks to be tested, and put on the lids.

Now place them in an incubator or water bath, kept at 38° C., and when the milk has reached this temperature add to each vessel ten drops of commercial rennet diluted with two or three cubic centimetres of boiled water. Shake or rotate the vessels gently so as to mix the rennet with the milk, and when curdling is complete, allow them to stand twenty minutes. Cut the curd in each into small pieces with a separate sterilized knife, or with one which is sterilized *each time before use* by dipping in boiling water, and cover up for half an hour. Then open the vessel and smell; note pleasant or disagreeable odour.

Pour off the whey, cover up, and leave for an hour; then note odour again, and pour off any whey still left.

Repeat observation every two hours.

After eight or twelve hours, take out the curd from each and cut it in two with a sharp knife.

Note the presence or absence of oval or round bubbles, or pin-holes, and the odour of the curd.

Large holes of irregular shape are due to want of close packing of the curd after being cut.

Ex. 163.—Inoculate two tubes of whey-gelatine with *Bact. lactis aerogenes*, or other organism isolated from spongy curd.

Incubate one of them at 22° C.; keep the other in a cool place at 15° C. or lower.

Note after twenty-four to twenty-eight hours the presence or absence of gas bubbles in each.

Ex. 164.—Take two tubes of whey-gelatine; to one of them add .1 per cent. of potassium nitrate.

Now inoculate both with *Bact. lactis aerogenes* or similar gas-producing organism.

Inoculate at 22° C.

Do colonies appear equally in both tubes?

In which are gas bubbles most abundant?

Test both at intervals of six hours for *nitrites* by method described in Ex. 74.

(ii) **Bitter Cheese.**—The production of bitterness in cheese is usually due to the work of micro-organisms.

The putrefactive bacteria may cause this trouble to arise, and some of the organisms noted as being responsible for bitterness in milk (p. 283) have been found to induce the objectionable flavour in cheese, although bitter milk does not necessarily make bitter cheese.

Freudenreich's *Micrococcus casei amari* was found to be one of the causes of the development of the objectionable flavour in Emmenthal cheese.

In 1901 Harrison isolated a species of *Torula* (p. 396) which he determined was the cause of serious trouble in one of the leading cheese factories in Canada. The organism, which he named *Torula amara*, produced an unpleasant bitter flavour in milk and also in cheese prepared from it.

It was traced to the leaves of certain species of maple which grew near where the milk-cans were stored. Grown in beer wort, the cells are oval, from 7.5 to 9 μ long, and, like most yeasts, occur singly or in short chains and small clumps. Inoculated into sterile milk the bitter taste develops in five or six hours at 37° C., with the formation of considerable quantities of gas and very slight acidity.

Cultures give the buiret-reaction of peptones.

Cheese made from milk inoculated with the *Torula* were bitter.

Eckles in 1905 isolated a small bacterium which was found to be the cause of bitterness in Cheddar cheese, the objectionable taste often developing in a few days.

The organism is a short rod, .8 to 1 μ long and .6 μ broad; when grown in whey it assumes the form of an oval coccus, .7 μ long, .6 μ broad.

It stains by Gram's method, and liquefies gelatine, the colonies being small and white at first, afterwards yellowish. Grown on agar at 37° C. for twenty-four hours, the colonies are very small, resembling those of *Str. lacticus*; deep colonies are yellow, those on the surface almost colourless.

Milk is coagulated by it in twenty-four hours at 37° C., the whey becoming straw-coloured and intensely bitter.

CHAPTER XXV.

MOULDS AND YEASTS.

MOULDS, yeasts, and other minute fungi are almost as widely distributed as bacteria, and the student of bacteriology is constantly meeting with them both as impurities in his cultures, and upon butter, cheese, and other materials with the study of which he may be largely concerned.

Some of these fungi are of great technical importance, and there is no doubt that many of the yeasts and moulds found in the soil, in manures, and in the interior and on the outside of cheese and other dairy products play considerable part in the characteristic chemical changes which go on in these materials.

The complicated chemical fermentations occurring in the putrefaction and decay of manure and organic matter in the soil, as well as in the "ripening" of cheese and other foods, are often the result of the symbiotic or combined activity of moulds, yeasts, and bacteria working simultaneously.

The present chapter deals with the chief morphological characters of the common kinds of moulds and yeasts.

Moulds.

1. **The genus Penicillium.**—One of the most frequently encountered small fungi, is common blue mould (*Penicillium glaucum*, Brefeld). The body of the plant is composed of

fine cottony filaments termed *hyphæ*. Each hypha is a transparent tube-like structure filled with colourless protoplasm, in which are vacuoles or spaces filled with water containing various substances in solution. In the younger parts of the hyphæ the protoplasm, in which small oil-globules are frequently abundant, fills the whole tube, but in the older portions it is found only as a thin lining on the inside of the hyphal filament.

Growth goes on at the top of each hypha.

Placed across the hyphæ of *Penicillium* at irregular intervals are thin transverse partitions or *septa*, and these are present in all except the lowest orders of fungi.

In most of the common moulds the body of the plant may be divided into two parts, namely :—

(1) The vegetative portion, named the *mycelium* or *spawn*, consisting of a tangled mass of hyphæ, which is generally embedded in the bread, cheese, or other material from which the fungus obtains its food.

(2) A more or less specialized portion, which bears the reproductive organs.

Reproduction among fungi is carried on by means of single-celled structures termed *spores*.

In Blue Mould these are borne in chains on *sterigmata*, which are arranged in a whorled pencil-like fashion at the ends of erect fertile hyphæ, as depicted in Fig. 51. Spores produced at the end of hyphæ and not enclosed in spore cases are frequently spoken of as conidia.

Some, if not all, of the species of *Penicillium*, have the power of forming fruiting bodies, inside which are spores of another type (ascospores): for further details upon these structures the student is referred to special text-books on fungi.

A number of apparently different species or varieties

of *Penicillium* are known, the following being, perhaps, the most important:—

(i) **P. glaucum**, Brefeld.—This species is found on bread, jam, fruit, and almost all kinds of decaying organic material; to its presence are due the characteristic blue veins in a ripe Stilton or Dorset cheese.

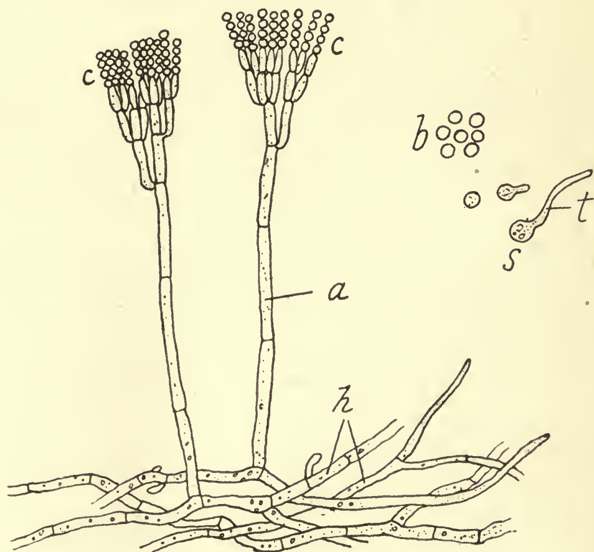


FIG. 51. A common "mould," *Penicillium glaucum*, Bref. *h* Hyphæ forming part of its mycelium; *a* erect hypha bearing conidia *c* in chains, *b* detached conidia, at *s* germination of a conidium has taken place, *t* germ-tube, the beginning of a new mycelium. (Enlarged 500 diameters.)

The mycelium and hyphæ in a young state are white and cotton-like; later, when the conidia are formed, the fungus becomes a greenish-blue colour.

The sterigmata are from 8 to 13 μ long and 3 to 4 μ broad. The conidia are round, smooth, and usually from 3 to 4 μ in diameter, with a greenish tinge.

The fungus grows best about 25 to 27° C.

(ii) **P. camemberti**, Thom.—A form or species of *Penicillium*, associated with the ripening of Camembert cheese, appears to be closely related to *P. glaucum*, but differs from it in some of its features. It is probably the same as *P. album* of Epstein, and *P. candidum* described by Rogers as occurring on Brie cheese.

At first its hyphæ are very white; in the early conidial stages it has a greyish-green tinge, becoming greyish-white later.

The sterigmata, 2 to 4 usually together, are 8 to 11 μ long, 2 to 3 μ broad; the conidia in a young state are sometimes slightly elliptical, but ultimately round and smooth, from 4.5 to 5.5 μ in diameter and yellowish-green when ripe.

The casein of milk is peptonized by it without coagulation.

(iii) **P. brevicaule**, Sacc., differs somewhat from the ordinary types of *Penicillium* in having its spores sometimes placed directly on the mycelial hyphæ instead of on special sterigmata. At first the fungus is white, but later turns brownish-yellow or brown; we have seen a deep orange form on the "coats" of Stilton cheese.

The sterigmata are comparatively few, usually bearing single terminal round conidia, which, when ripe, are 5 to 7 μ in diameter, with warted surfaces.

Weigmann and Wolff found a form of this fungus in the dung of cows fed largely on turnips (see p. 355).

Ex. 165.—Place a piece of mouldy Stilton cheese, or mouldy bread, on some damp blotting-paper in a deep Petri dish. Leave for a day or two until active growth of the mould commences.

(*a*) Remove a portion of the blue mould with a pair of

forceps; place it on a dry slide and examine it with a low power of the microscope or a strong hand lens.

(*b*) Mount some in a drop of water, and after tearing the hyphæ apart with a couple of needles, examine it first with a low power, noting the large number of separate conidia and tangled hyphæ.

Then examine it with a high power, and make drawings of the conidia and the hyphæ with their contents and septa.

(*c*) Take a fresh piece of the mould and dip it in a watch-glass containing absolute alcohol, to which a drop or two of strong ammonia have been added.

Then transfer successively to a series of watch-glasses containing 75 per cent., 50 per cent., 25 per cent., and 10 per cent. alcohol respectively, leaving it thirty seconds in each.

Mount it in a drop of water, and examine it for conidiophores with unbroken brush-like sterigmata and chains of conidia.

Make drawings illustrating the arrangement and structure of the several parts observed.

Measure the conidia and sterigmata.

Ex. 166.—Place a piece of Camembert cheese under a bell-jar, or in a Petri dish, on some damp blotting-paper. After a few days, examine the moulds which grow on it, in the manner described in the previous experiment.

Make drawings and measurements of the parts of the fungi observed: compare with *P. glaucum*.

Ex. 167.—Examine the yellowish, orange, or brownish patches on the outside of any Stilton cheese available for *P. brevicaule*.

2. The genus Aspergillus.

An almost equally ubiquitous group of fungi are included in the genus *Aspergillus*. They are closely related to the genus *Penicillium*, and, like the latter, the

hyphæ and conidia of several species are grey or bluish-green in colour.

The erect spore-bearing hyphæ or *conidiophores* do not branch, but end in swollen, rounded apices. The sterigmata,* also, are usually simple and unbranched, and bear chains of round or oval conidia (Fig. 52). These fungi produce small round yellow or brownish perithecia or fruits containing asco-spores, which are more frequently met with, perhaps, than the corresponding organs of *Penicillium*.

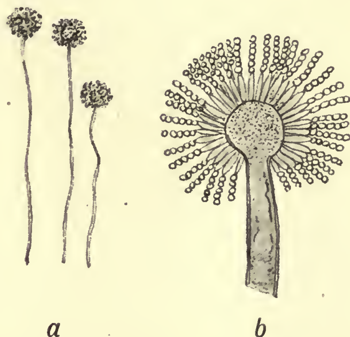


FIG. 52.—*Aspergillus glaucus*, Link.
a Conidiophores under low magnification (× 60).
b More highly magnified (× 300).

Common representatives are :—

(i) **A. glaucus**, Link., is a very widely distributed fungus on old bread, jam, and all kinds of decaying organic substances. The creeping hyphæ are cottony, branched, indistinctly septate: the fertile hyphæ are usually without septa, inflated at the end to about 60 μ in diameter, and covered with small sterigmata bearing chains of round conidia about 8 to 10 μ in diameter. The conidia are at first colourless, but later become glaucous or greyish-green, and finally brownish in colour: they frequently have very minute wart-like projections on their outer surfaces.

The perithecia which are commonly found on many organic substrata are round, bright-yellow bodies 100 to 200 μ in diameter, and containing asci with 5 to 8

colourless oval ascospores 7 to 10 μ long and 5 to 8 μ broad: later they become brown.

The ascigerous or perithecial stage of the fungus is known as *Eurotium herbariorum*, Link.

(ii) **A. flavus**, Link., when fully developed, is a yellowish or tawny-coloured fungus. It occurs on bread, dried plants, and various organic substances, and is found as a saprophyte in the human ear sometimes.

The apical inflated part of the conidiophore measures 30 to 40 μ across: the conidia are round, minutely warted, yellowish, and 5 to 7 μ in diameter. It grows best at about 37° C.

(iii) **Aspergillus niger**, Van Tieghem, is a common mould with dark brown or black globose heads of conidia. The hyphæ are colourless, or practically so, but the conidia are a smoky-brown tint, round, and 3 to 5 μ in diameter. The inflated end of the conidiophore is about 80 μ across, and bears a large number of delicate *branched* sterigmata, on which latter account the fungus is more correctly included in the genus *Sterigmatocystis* under the name *S. niger*, Cramer.

(iv) **A. Oryzæ**, Cohn, is utilized in Japan for the preparation of Saké, a kind of wine made from rice.

Ex. 168.—Procure a specimen of *Aspergillus glaucus* and examine it both with low and high power according to the method described in Ex. 165.

Note the shape and arrangement of the conidia: compare with *Penicillium glaucum.*

Make drawing of the conidiophore, its inflated apex, and the conidia, and measure the diameter of each.

3. **Cladosporium herbarum**, Link.—One of the dark-coloured saprophytic moulds met with everywhere upon

decaying organic matter is *Cladosporium herbarum*, Link., a conidial stage of some ascomycetous fungus. It frequently produces dark olive-brown patches upon damp paper, wood, barley, and other grains, dead leaves of all kinds, and vegetable tissues generally. A form of it, *Cladosporium butyri*, occurs sometimes upon rancid butter (p. 354) and other dairy products.

The hyphæ, densely woven into a blackish-olive stratum, often become divided into very short, almost rectangular, lengths by transverse partitions, which pieces may ultimately separate and germinate in the same manner as a conidium or spore.

The true conidia arise singly or in very short chains of two or three members at the tips of sparingly branched hyphæ: they are at first pale in colour but later become a dark olive-brown colour. In size and form they are extremely variable, some of them being oval and others oblong or cylindrical, usually smooth, simple, or with one or two septa: most commonly they are oval, from 12 to 20 μ long and 5 to 8 μ broad.

Ex. 169.—Procure and examine a specimen of *Cladosporium herbarum*.

Note the colour of the hyphæ and their numerous septa.

Observe also the form and size of the conidia.

4. **Oospora lactis**, Sacc.—This organism, most commonly known by its earlier name *Oidium lactis*, Fres., forms a snow-white velvety covering upon cream and butter and on the outsides of many of the softer kinds of cheese. The sterile hyphæ are closely interlaced with each other and spread over considerable areas when the conditions for nutrition are suitable. In liquid media they frequently break up into short lengths very closely

resembling the conidia of the fungus, except that the ends preserve their rectangular outline for some time, becoming rounded only when they begin to germinate (Fig. 5).

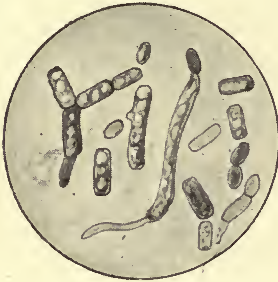


FIG. 53.—*Oospora lactis*, Sacc. (*Oidium lactis*, Fres.).

The fertile hyphæ bear conidia, some of which are globose or oval, others longer and more or less cylindrical from 18 to 21 μ long and 5 to 8 μ broad.

Oospora lactis grows well upon acid media, especially those containing lactic acid, which it oxidizes readily. Acid gelatine is liquefied and the casein of milk is peptonized by it: the optimum temperature for its growth is about 20° C.

Ex. 170.—Procure a piece of rancid butter, or keep a piece of butter on damp blotting-paper in a Petri dish for three or four weeks.

From the outside of either scrape off a small portion: work it gently in a mortar with a little water and inoculate a tube of melted lactose gelatine with a drop of the mixture. Pour into a Petri dish and incubate at 20° C.

Examine any white stellate colonies for *Oospora lactis*.

When found, make drawings of the conidia and hyphæ.

Inoculate a tube of sterile milk with the fungus and incubate at 20° for two days: examine a drop of the milk for the fungus and note the rectangular outline of pieces of the hyphæ.

Stain some with weak gentian violet.

The fungus may sometimes be obtained on cream when it is left in a beaker undisturbed for a time.

Ex. 171.—Grow *Oospora lactis* in sterile litmus milk.

Does the latter become acid or alkaline?
Is casein precipitated or dissolved?
Note the odour of the milk.

5. **The genus Mucor** embraces another group of small moulds, one of the commonest representatives being *Mucor Mucedo*, L.

(i) **Mucor Mucedo**, L., leads a saprophytic life, and is very commonly found as a white fluffy mould upon damp bread, jam, decaying fruit, and other organic materials, especially those containing sugars and starch.

It is frequently seen on damp horse-manure, and also occurs upon butter and cheese, in which it sets up decomposition of the fats and proteins.

The mycelium differs from that of the previously mentioned fungi in being unseptate, or without divisions across its hyphæ. The fertile hyphæ are erect, unbranched, and bear on their ends round structures, 100 to 200 μ in diameter, termed *sporangia*, inside which are produced a large number of smooth, transparent, oval spores, usually about 12 to 18 μ long and 6 to 8 μ broad (Fig. 54).

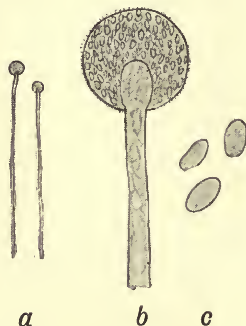


FIG. 54.—Sporangia of *Mucor Mucedo*, L.
a Magnified 5 diameters.
b Magnified 400 „
c Spores (×500).

The apex of the sporangial hyphæ or *sporangiophore* extends some way into the interior of the sporangium as a cylindrical or ovoid bladder-like projection termed the *columella*.

Most species of *Mucor* give rise to a sexually-produced kind of spore—a thick-walled, black-warted *zygospore*—

which usually appears in the substratum on which the fungus is growing. For further details of its origin and structure the student must refer to text-books of mycology.

(ii) **Mucor pyriformis**, Fischer, is another species found on damp, bruised, and decaying apples and other fruits, from the sugars of which it appears to be able to produce small quantities of sweet-smelling esters and citric acid.

It possesses large brownish sporangia 350 to 400 μ in diameter, in which are oval spores 6 to 9 μ long and 4 to 5 μ broad: the columella is pear-shaped.

(iii) **Mucor racemosus**, Fres., is a very widely distributed species upon fruit, bread, and vegetable tissues of all kinds, and found also upon horse-dung, cheese, and many dairy products.

Its sporangia are small, from 20 to 60 μ in diameter, yellowish or brownish in tint, and borne either upon simple unbranched hyphæ or on sporangiophores with short racemose branches (Fig. 55).

FIG. 55.—*Mucor racemosus*, Fres. ($\times 5$).

The spores are smooth and elliptical, 5 to 6 μ long and about 4 μ broad; the columella is egg-shaped.

The fungus grows best at 20 to 25° C., and is of especial interest on account of its power of fermenting sugar solutions, with the production of considerable amounts of alcohol.

This it largely accomplishes by means of sprouting yeast-like cells which originate from submerged hyphæ or portions of the mycelium.

Ex. 172.—Place a piece of fresh horse-dung under a bell-jar

on wet blotting-paper; leave until mouldy. Various species of fungi make their appearance. Look especially for *Mucor Mucedo* or *M. racemosus*, known by their round sporangia, which look like small pin-heads on the ends of thin white stalks.

When obtained, take up with fine pointed forceps a very small portion, and transfer to a drop of water on a slide. Cover with cover-slip, and examine the hyphæ bearing the sporangia; note the absence of septa in the hyphæ.

The sporangia, with ripe spores in them, burst immediately when placed in water.

Examine the oval spores with a high power.

To see the spores within the sporangia, take the hyphæ bearing

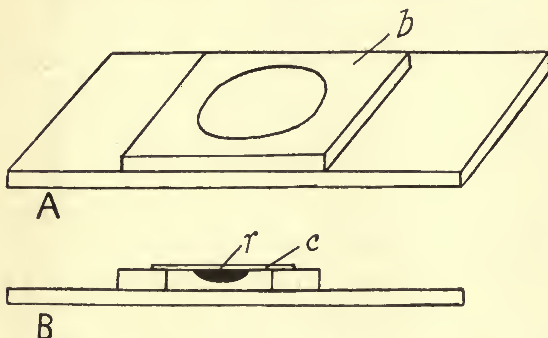


Fig. 56.—Moist chamber for observing the germination and growth of spores in hanging-drops. *b* Cardboard with circular hole punched in it, resting on ordinary glass slide. *B* Section of *A* with cover-slip (*c*) and hanging-drop (*r*) in position.

them with forceps and mount in alcohol on a slide: then examine quickly with a high power.

Ex. 173.—In order to observe the germination of these and other spores, and watch their subsequent development for a time, a moist chamber, prepared as follows, is necessary:—

Place fifteen or sixteen pieces of blotting-paper on one another and punch out, or cut out, a round or square hole slightly less than the size of a three-quarter inch cover-slip. Cut the blotting-paper afterwards so as to fit on a slide, as in *A*, Fig. 56.

A piece of stout cardboard, cut in a similar manner, may be used instead of blotting-paper.

In the centre of a cover-slip place a small drop of water, or a drop of a very dilute extract of French plums which has been boiled. Shake or otherwise transfer the spore to be germinated into the drop of water, and then place the cover-slip over the hole in the cardboard with the drop hanging downwards, as in *B*, Fig. 56.

Keep the whole on damp blotting-paper under a bell-jar.

The spores can be readily examined from day to day, even with a high power, through the glass of the cover-slip without moving or disturbing the latter.

Yeasts (*Saccharomyces* and *Torula*).

1. **Saccharomyces.**—The term yeast is applied to a large series of simple unicellular fungi which have the power of producing alcohol when grown in the juices of fruits, malt-extract or "wort," and other solutions containing sugars.

Those which are included in the genus *Saccharomyces* reproduce themselves in two ways, viz :—

(*a*) By the process of sprouting, budding, or gemmation.

(*b*) By means of specialized spores (ascospores).

Certain unicellular fungi, closely resembling yeasts in form and size, and in the sprouting process, but which do not form ascospores, are classified as belonging to the form-genus *Torula*.

In the vegetative state yeast plants consist of single oval cells, which under certain conditions may become considerably elongated so as to resemble hyphal filaments, but no true mycelium is formed by these fungi.

The yeast-cell has an elastic cell-wall filled with protoplasm in which are one or two large vacuoles.

When grown in sugar-solutions multiplication takes place by the process of budding, each cell sending out one or more "sprouts" or protrusions which grow to the size of the parent and then separate from the latter as new individuals.

The true yeasts when grown upon slices of potato or moist slabs of gypsum, with little nourishment, form spores, the protoplasm of each cell dividing into two or

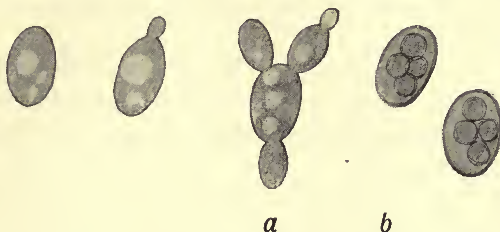


FIG. 57.—Beer Yeast (*Saccharomyces cerevisiæ*, Meyer), showing budding (*a*) and ascospore formation (*b*).

four portions which surround themselves with thin walls, the whole yeast plant thus becoming transformed into a spore-case termed an *ascus*, the spores within being spoken of as *ascospores* (Fig. 57).

Many species, races, and varieties of *Saccharomyces* are recognized: they all break down sugars into alcohol and carbon dioxide gas, forming at the same time small quantities of glycerine, succinic acid and other organic compounds. They hydrolyse or "invert" maltose and other di-saccharose sugars by means of the enzyme *invertase* which they secrete, but their power of producing alcohol is due to an enzyme *zymase* which can only be obtained from the cells by grinding them with sand or other fine powder and then subjecting the dead cells to high pressure.

Most of the races of yeast used in the manufacture of beer in this country carry on rapid fermentation at temperatures between 12 to 24° C., and are lifted to the surface of the liquid as a frothy scum by the bubbles of carbon dioxide set free: such are known as *top-fermentation yeasts*. Others, termed *bottom-fermentation* yeasts, and utilized for the production of lager beer at 5 to 10° C. produce alcohol more slowly, and accumulate as a muddy layer on the bottom of the vessel in which the fermentation takes place.

A great number of species and varieties of yeasts have been described: a few only need mention here.

(i) **Saccharomyces cerevisiæ**, Meyer. *Beer yeast.*—The typical form of this species, of which many varieties exist, is roundish oval: usually 1 to 4 ascospores from 3 to 6 μ in diameter, are produced in each cell the optimum temperature for their formation when grown on gypsum blocks is about 30° C. It ferments glucose, saccharose and maltose, but not lactose.

(ii) **S. pastorianus**, Reess., possesses oval, round and long sausage-shaped cells (Fig. 58). The ascospores are generally 1 to 4 in number, and from 2 to 4 μ in diameter. They are formed most rapidly at 27 to 28° C. It is a wild yeast which gives a bitter taste and unpleasant smell to beer. It does not ferment lactose.

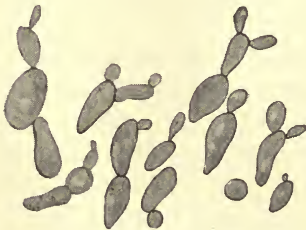


FIG. 58.—*Saccharomyces pastorianus*, Reess.

(iii) **S. ellipsoideus**, Reess., is one of the species or forms of yeast concerned in the manufacture of wines from grape juice: it occurs on the exterior of grapes,

plums, cherries and other fruits. The cells are usually elliptical in outline, but elongated forms are frequent.

The ascospores, 2 to 4 μ in diameter, are produced in about twenty hours at 25° C.

Like the above species it does not ferment lactose.

(iv) **S. flava lactis**, Kruger.—This form produces yellow colonies with rapid liquefaction on gelatine media: it was originally isolated from a "cheesy" butter which had a disagreeable odour of decomposing urine. Its cells are ellipsoidal and about 4 μ long. It ferments lactose as well as maltose and cane-sugars.

(v) **Saccharomyces fragilis**, Jörgensen, obtained from Kefir, ferments milk-sugar with production of a small quantity of alcohol.

It is a small oval species, sometimes with elongated cells: two to four oval ascospores are produced in about twenty hours when the cells are grown on gypsum blocks at 25° C.

Ex. 174.—Obtain a small piece of German yeast. Break it in pieces and stir it up in water in a tea-cup. Allow the yeast to settle and pour off the water. Mount in water a drop of the creamy yeast left at the bottom of the cup, and examine with a high power.

Draw a single cell and observe its cell-wall, protoplasm, and vacuoles.

Ex. 175.—Half fill a small tea-cup with water and dissolve in it a teaspoonful of sugar, put in a piece of German yeast about the size of a broad-bean and stand the whole in a warm place. When the yeast begins to accumulate on the surface of the water, take a very minute portion and mount it in water. Examine with a high power and sketch the budding or sprouting cells.

Ex. 176.—Smear some of the creamy yeast from Ex. 174 on

the cut surface of a slice of potato; place the latter on damp blotting-paper in a Petri dish and incubate at 20° C.

Scrape off daily a little of the yeast and mount in water. Examine with an $\frac{1}{8}$ objective for ascospores.

Ex. 177.—Take some plaster of Paris (or gypsum) and mix it with water in the proportion of eight volumes of the powder to three of water. Mould it rapidly before it sets into the form of short cylinders or truncated cones about an inch in diameter and $\frac{3}{4}$ of an inch high, pressing the upper surfaces against a sheet of glass to render them smooth. The material soon sets into hard blocks which should be sterilized by heating when dry in a hot-air sterilizer to 115° C. for one hour.

Smear some of the creamy yeast from Ex. 174 on the upper surface of a block, and place the latter in a deep Petri dish. Pour in boiled water until the block is half immersed: then place the whole in an incubator at 20° C., and examine small portions of the yeast in twenty-four to forty-eight hours for ascospores.

Ex. 178.—Repeat the above experiments, using ordinary brewers' yeast instead of German yeast.

Ex. 179.—Inoculate gelatine plates with a drop of water in which yeast has been distributed: isolate the yeasts from any accompanying bacteria and grow them upon gelatine, agar, and potato, noting the form and character of the pure colonies.

Ex. 180.—Prepare cover-glass films of yeasts from cultures in beer-wort or other liquid media, and stain with Löffler's methylene blue or weak gentian violet; wash, dry, and mount.

Ex. 181.—Mix some of the gypsum-block cultures showing ascospores with a little water: spread a small drop on a cover-glass, and after air-drying, fix and stain with carbol-fuchsin for two minutes. Then wash in water and in alcohol to remove colour from all parts of the cells except the spores: counterstain with Löffler's methylene blue for three minutes.

2. **Torula.**—The genus *Torula* embraces single-celled

budding or sprouting fungi, which form neither endospores nor true hyphæ or mycelia (Fig. 59). In morphology and physiological function they are very closely allied to the yeasts proper, differing from the latter only in the absence of ascospore formation. Some of them may belong to the genus *Saccharomyces*; others, which are very imperfect, may possibly be budding or sprouting conidia of certain higher fungi.

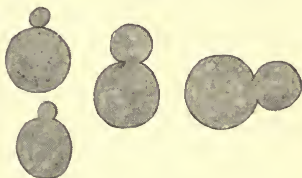


FIG. 59.—*Torula* cells.

The various forms of *Torula* have rounded, oval, or somewhat elongated cells like the yeasts previously described, and may occasionally be met with united into chains of variable length. Many of them are colourless, others are coloured a reddish-pink or rosy tint. Some systematists classify the colourless and highly coloured forms in the genus *Oospora*, reserving the generic name *Torula* for those of brown, olive, and blackish hue.

These minute fungi are ubiquitous, being found in water, in the dust of the air, in the upper layers of the soil, and among decaying vegetable refuse. Some of them are particularly abundant upon cheese, butter, and other dairy products, and are able to grow and produce acids in milk, with or without coagulation of the casein. In Kefir and Mazun they are present, and there is some evidence for the belief that they are of importance in the development of the characteristic flavours of certain kinds of cheeses and milk beverages.

Very few of them are known with a sufficient degree of accuracy and completeness to warrant special description here.

A form named by Harrison, *Torula amara*, was found to be the cause of a bitter taste in milk and cheese, and Rogers mentions a variety which gave an objectionable flavour to butter which was packed in wooden casks.

Ex. 182.—Isolate and study the various kinds of *Torula* occurring—

(*a*) On the rind of Stilton or soft cheeses;
(*b*) In soil;
(*c*) In farmyard manure.

Grow them on gelatine and agar media, in plain and sugar-bouillon, and in milk.

Note the form and colour of their colonies, their liquefying action on gelatine, their power of coagulating or rendering milk acid or alkaline, and their action on bouillon containing sugar.

Differentiate them from *Saccharomyces*.

Try growth on gypsum blocks.

Bacteriological apparatus of all kinds can be obtained from Messrs Baird & Tatlock, Cross Street, Hatton Garden, London, E.C.

TABLES.

Measures of Length.

English.

1 inch			=	25.4 millimetres.
1 foot	=	12 in.	=	30.8 centimetres.
1 yard	=	3 ft.	=	91.44 ,,
1 chain	=	22 yds.	=	20.11 metres.
1 mile	=	1760 yds.	=	1.61 kilometres.

Metric System.

1 millimetre	=	.001 metre	=	.0393	in.	or about	$\frac{1}{25}$ in.
1 centimetre	=	.01 ,,	=	.393	,,	,,	$\frac{3}{8}$ in.
1 decimetre	=	.1 ,,	=	3.937	,,	,,	4 in.
1 metre	=	1 ,,	=	39.370	,,	,,	$3\frac{1}{4}$ ft.
1 decametre	=	10 metres.					
1 hectometre	=	100 ,,					
1 kilometre	=	1000 ,,	=	1094	yds.	,,	.621 mile.

Measures of Area.

English.

1 square inch.				
1 ,, foot	=	144 sq. in.		
1 ,, yard	=	9 sq. ft.	=	8 milliares.
1 acre	=	4840 sq. yds.	=	40.46 ares.

Metric System.

1 sq. centimetre	=	.155 sq. in.		
1 are	=	119.6 sq. yds.	=	3.9 perches.
1 hectare	=	2.47 acres.		

Measures of Capacity.

English.

1 minim			=	.059 cubic centimetre.	
1 fluid drachm	=	60 minims	=	3.55 ,,	centimetres
1 ,, ounce	=	8 drachms	=	28.35 ,,	,,
1 pint	=	20 ounces	=	567.9 ,,	,,
1 quart	=	2 pints	=	1.137 litres.	
1 gallon	=	4 quarts	=	4.546 ,,	

1 fluid ounce weighs 1 ounce avoirdupois.

Metric System.

1 cubic centimetre (1 c.c.) = 16.89 minims.

1 litre = 1000 c.c. = 1.76 pint = .22 gallon = 35.2 fluid ounces.

1 gallon of water = 227.27 cubic inches and weighs 10 lbs. at 62° F.

Measures of Weight.

Avoirdupois.

1 dram			=	1.77 grams.
1 ounce	=	16 drams	=	28.35 ,,
1 pound	=	16 ounces	=	453.59 ,,
1 hundredweight (cwt.)	=	112 lbs.	=	50.8 kilograms.
1 ton	=	20 cwts. = 2240 lbs.	=	1016 ,,
1 ounce	=	437.5 grains.		

Troy.

1 grain			=	.0648 gram.
1 pennyweight (dwt.)	=	24 grains	=	1.55 grams.
1 ounce	=	20 dwts.	=	31.10 ,,
1 pound	=	12 oz.	=	373.2 ,,
1 ounce	=	480 grains		

Metric System.

1 milligram	=	.001 gram	= .015 grain (Avoir).
1 gram	=	1 ,,	= 15.43 grains ,,
1 kilogram	=	1000 grams	= 2.2 pounds ,,

The circumference of a circle = πd.
The area of a circle = πr^2.
The surface of a sphere = $4\pi r^2$ or πd^2.
The volume of a sphere = $\frac{4}{3}\pi r^3$.

π = 3.1416.
d = diameter.
r = radius

Comparison of the Centigrade and Fahrenheit Thermometer Scales.

(i) To convert degrees Centigrade to Fahrenheit, multiply by 9, divide by 5, and add 32.

(ii) To convert degrees Fahrenheit to Centigrade, subtract 32 and multiply the remainder by 5 and divide by 9.

°C	°F.	°C.	°F.	°C.	°F.	°C.	°F.
0 =	32	26 =	78.8	51 =	123.8	76 =	168.8
1	33.8	27	80.6	52	125.6	77	170.6
2	35.6	28	82.4	53	127.4	78	172.4
3	37.4	29	84.2	54	129.2	79	174.2
4	39.2	30	86.	55	131.	80	176.
5	41.	31	87.8	56	132.8	81	177.8
6	42.8	32	89.6	57	134.6	82	179.6
7	44.6	33	91.4	58	136.4	83	181.4
8	46.4	34	92.2	59	138.2	84	183.2
9	48.2	35	95.	60	140.	85	185.
10	50.	36	96.8	61	141.8	86	186.8
11	51.8	37	98.6	62	143.6	87	188.6
12	53.6	38	100.4	63	145.4	88	190.4
13	55.4	39	102.2	64	147.2	89	192.2
14	57.2	40	104.	65	149.	90	194.
15	59.	41	105.8	66	150.8	91	195.8
16	60.8	42	107.6	67	152.6	92	197.6
17	62.6	43	109.4	68	154.4	93	199.4
18	64.4	44	111.2	69	156.2	94	201.2
19	66.2	45	113.	70	158.	95	203.
20	68.	46	114.8	71	159.8	96	204.8
21	69.8	47	116.6	72	161.6	97	206.6
22	71.6	48	118.4	73	163.4	98	208.4
23	73.4	49	120.2	74	165.2	99	210.2
24	75.2	50	122.	75	167.	100	212.
25	77.						

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