Thermophilic bacteria : (second paper) / by Allan Macfadyen, M.D., and Frank R. Blaxall, M.D.

THERMOPHILIC BACTERIA.

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(*Second Paper.*)

THE first observations we made upon bacteria growing at high temperatures were published in the *Journal of Pathology and Bacteriology* in 1894.[1] These observations showed that such organisms were of great variety and widely distributed. They did not occur as isolated examples, but constituted a group to which the term of thermophilic bacteria might be applied.

At that time the subject of thermophilic bacteria had been but little investigated—beyond the isolated observation on the bacillus thermophilus by Miquel, and the partial study of some similar forms by Globig.

Some months after the appearance of our paper a research on similar lines was published by L. Rabinowitsch.[2] This paper, which will be referred to subsequently, contained practically a confirmation of our results, as well as to a certain extent a repetition of the theories we had brought forward regarding the possible conditions for the growth of such organisms. There have also been a few other communications published, and these will be referred to in the course of the paper. At the same time, there has been no prolonged investigation of the group as a whole. The subject appeared of such interest, as well as the problems connected with it, that we continued its investigation. The following results have confirmed our previous investigations, and have also brought out some new facts. The experiments extended over a considerable period of time (two years), and were

made upon a large amount of material, so that there might be adequate data for any conclusions reached. The details also are fuller with regard to the cultural and other peculiarities of the organisms. In our previous paper the conclusion we had reached was that there is a widely distributed group of organisms in nature which find their optimum temperature of growth between 55° and 65° C. At that time we isolated from various sources and described fifteen forms which not only grow best at these high temperatures, but also retain all the activities of the living cell. The further details will be found in the paper cited.

The wide distribution of these organisms is of a very striking nature. The material examined by us was collected from the most varied sources and cultivations made at 55° to 65° C.

The organisms were found in Thames water, Thames mud, sewage water; and in sea water from Plymouth a thermophilic bacillus was detected. They were also detected in the surface and deep layers of the soil, and in dust from the streets and house roofs, as well as in the intestine of man and the horse. They were present in the digestive tract of ducks, fowls, mice, rabbits, guinea-pigs, worms, frogs, and snails. Manure heaps, as one would expect, contained large numbers. Amongst fish they were found in the digestive tract of goldfish, carp, eel, and whiting, as well as in oysters. They were present in straw and in all samples of ensilage examined. They were not detected in tap water or in sputum. The results were also negative in the case of a thermal spring at Bath (T. 118° F.). A sample of volcanic dust from Japan, accidentally obtained, gave no growth.

Amongst other experiments a freshly dead mouse was placed with a little water in a sealed jar, and kept at 65° C. The mouse became mummified, but no putrefactive decomposition occurred. The ordinary putrefactive bacteria were killed by the heat, and the thermophilic organisms appeared incapable of producing a decomposition of the animal. Further, the ordinary bacteria of putrefaction were not capable of adapting themselves to a thermophilic temperature. They are excluded from any chance of being mixed up with the

thermophilic bacteria. A large number of organisms were isolated, for which the optimum temperature of growth is 55° to 65° C. They possess this peculiarity as their cardinal feature. The existence of these organisms may be demonstrated in the following simple manner :—A sample, say of soil, is placed on sloping agar, and incubated at 65° C. A rapid development occurs, and in fifteen to seventeen hours the whole agar surface is covered with a growth. From this growth agar plates of various dilutions are made. Colonies of differing character will appear, indicating the presence of more than one kind of thermophilic organism.

Thus we may observe an anthrax-like colony, a colony resembling B. figurans and smaller round whitish colonies on the same plate. The plates are frequently entirely covered with a thin filmy growth, which microscopically appears to be a pure culture of one organism. This frequently prevents one obtaining subcultures of deep colonies in the agar without contamination by the surface growth. A larger series of plate cultures did not always overcome this difficulty, and the subcultures were frequently found to contain mixed growths. This tendency to diffuse surface growth of one species proved troublesome, and had to be checked. The addition of various substances to the nutrient agar was tried with this end in view, *e.g.*, 2 per cent. sodic chloride, glycerine, &c. The results, however, were not satisfactory. After some experimenting we obtained in potato agar a soil which gave good results. The organisms grew on this soil as isolated colonies, and remained discrete, and it was possible to obtain readily pure subcultures of the organisms. This soil proving the best, all the subsequent isolation experiments were carried out with it. One need not fear a drying up of the nutrient medium at these high temperatures, as the growths that occur are so rapid. A large number of thermophilic colonies were in this way obtained from soil, &c.

The potato agar is prepared as follows :—Potatoes are first steamed, peeled, and pounded. To 100 grammes of potato is added one litre of tap water, and the mass is steamed for half an hour and filtered. To the filtrate 1·5 to

2 per cent. of agar is added, and the whole autoclaved for fifteen minutes. It was found an advantage to add 1 per cent. of salt. After neutralisation with soda and further steaming, the potato agar is filtered into test-tubes and sterilised once more. It will be seen that this is practically a carbo-hydrate soil; it will be referred to as salt potato agar.

From the primary colonies obtained on this soil subcultures were made on the ordinary culture media, on which as a rule thermophilic bacteria readily grow. The organisms can also be readily cultivated from generation to generation on this soil at high temperatures—a further proof of their thermophilic character.

As regards cultural peculiarities, we tested other soils, such as slices of carrot, beet, and turnip, with unfavourable results. In many instances no growth occurred, or it was of a diffuse character. Naegeli's nutrient salt solution did not furnish any perceptible growth, but growths appeared upon the addition of a trace of peptone broth. Globig, and also Rabinowitsch, used the potato for isolation purposes. We did not, after testing the point, select potato for this purpose, because the growth of the organisms is often slow; some will not grow on it, and the colonies that do occur are frequently so mixed that it involves much trouble to separate pure cultures.

Notwithstanding frequent attempts, several of the organisms could not be made to grow upon simple potato. The potato, though used by the above observers, is not a suitable soil for isolating all the varied forms of these bacteria that undoubtedly exist. Those that do grow on potato generally give characteristically pigmented growths, easily differentiated by the pigment produced, and especially the forms occurring in soil. For example, a sample of soil sown directly on potato gave the following pigmented growths:—Canary yellow, white crusty, umber, cream, dull brick, buff, and brownish coloured growths. It is interesting to find such bright pigment production occurring at 56° C., and it is another instance of the full vitality of the organisms at this temperature.

After these preliminary experiments we selected salt potato agar for isolation purposes instead of the ordinary

agar originally employed. From the colonies and their sub-cultures microscopical specimens were made, and showed without exception bacillary forms. At times long thread-like and interlacing filaments were visible, but we were unable in any instance to demonstrate genuine branching forms. Their growth was also studied on gelatine, serum, potato, milk, &c., and in this way many useful differentiations were brought out. It became speedily apparent that we could not hope to describe exhaustively all the forms that presented themselves, and a process of selection had accordingly to be adopted. We selected frequently recurring organisms as characteristic and typical of the group, and their morphology and biology were more closely studied than had been done in our previous paper. It is proposed to give a description of fourteen forms, and some illustrations are given of the most characteristic colonies. It has not been thought advisable to give them names, but to simply distinguish the organisms as Bacillus I., II., III., &c.

The various organisms stain with the ordinary dyes, but, on the whole, carbol. methylene blue was found to give as good results as any. It was usually found advisable, before staining, to treat the cover-slip specimen with dilute acetic acid. This cleared away a zoogloea-like membrane, which often interfered with clear staining, and such specimens gave better results.

The following is a description of the colonies as they appeared on salt potato agar, along with the microscopical peculiarities, &c. The subsequent table gives the main cultural appearances of the growths obtained, indol reaction, &c.

Morphology, &c., of the Thermophilic Bacteria.

In all instances the description of the colonies refers to a twenty-hours' growth on salt potato agar at 55°-60° C. The hanging drops were made from six-hours' cultures, to avoid sporing forms :—

Bacillus I. (from animal dejecta).—Marked surface growth, colonies 8 to 10 mm. in diameter, yellowish white, resemble small ball of cotton-wool with teased out margin. × 50,

appear yellowish brown, anthrax-like, tendril-like runners on agar surface, forming loops and convolutions (fig. 1).

Organism non-motile, rods 3 to 4 times as long as broad; average, L. 3 to 4 μ, Br. 1 μ. Form long chains, frequently apposed and parallel; also looped and twisted with pseudo-branching arrangement; ends rounded, in chains frequently flattened.

Gram:—Negative; spores towards centre of slightly swollen rod.

Bacillus II. (from animal dejecta).—Surface colonies about 2 mm. in diameter, dull white, round, smooth contour. × 50, appear yellowish brown, finely and uniformly granular, slightly wavy at margin (fig. 2).

Organism non-motile, rounded ends; L. 4 to 6 μ, Br. 1·25 to 1·5 μ.

Gram:—Positive, oval spore towards one end of rod, somewhat larger than bacillus, and giving a "fishfloat" appearance.

Bacillus III. (from soil).—Small pinhead colonies, dull whitish colour. × 50, appear yellowish brown, finely fringed margin, finely granular with delicate wavy linear markings running towards periphery.

Organism non-motile, large rods, about five times longer than broad, ends rounded, tendency to form short wavy chains; no false dichotomy; L. 6 to 8 μ, Br. 1 μ.

Gram:—Positive; large, spoon-shaped terminal spore.

Bacillus IV. (from ensilage).—Dull white, spherical colonies, about 1 μ in diameter, with mottled appearance. × 50, appear round, pale yellow, coarsely granular, and smooth contour (fig. 3).

Organisms present oscillatory movements, otherwise non-motile; a short rod; L. 3 to 4 μ, Br. 1 μ, ends rounded, single and in pairs; no chains.

Gram:—Positive; small oval spore towards slightly swollen end, and occupying about three-fourths of bacillus.

Bacillus V. (from Thames mud).—Spherical, yellowish white colonies with regular margin, about 4 mm. in diameter. × 50, appear dark brown and finely granular in central zone, which occupies three-fourths of colony; outer zone is pale

yellow, coarsely granular, with ridged markings, and slightly teased out at margin (fig. 4).

Organisms have oscillatory motion; otherwise non-motile; resembles Bacillus IV., but more slender, ends rounded, short wavy chains.

Gram:—Negative; spores freely, spore terminal and about same diameter as rod.

Bacillus VI. (from Plymouth seawater).—Dull white, and feathery colonies. × 50, are characteristic; irregular shape and of a fleece-like texture throughout; somewhat resemble finely teased out cotton-wool fibres in the agar (fig. 5).

Organisms non-motile, large slender rods, tendency to pointed ends and chain formation; L. 6 to 10 μ, Br. 1 μ.

Gram:—Negative; drumstick spore, giving "bonnet-pin" appearance.

Bacillus VII. (from soil).—Round, dry, feathery colonies, dull white enamel appearance, 4 to 12 mm. in diameter. × 50, appear brown and feathery, central markings, finely granular at margin, which likewise shows fluted markings and indentations (fig. 6).

Organism non-motile, rods about size of anthrax bacillus, ends slightly flattened, in pairs and short chains.

Gram:—Positive; large, spoon-shaped terminal spore.

Bacillus VIII. (from soil).—Yellowish white, spherical colonies. × 50, appear yellowish brown, "biscuit" appearance, distinct rimmed margin, contour finely serrated, colony is coarsely granular and shows three zones, an outer clear, a middle dark brown and a central light brown zone (fig. 7).

Organism, oscillatory, otherwise non-motile; rounded ends, and short curving chains; L. 4 to 8 μ, Br. 1 μ.

Gram:—Positive; oval central spore filling greater part of bacillus.

Bacillus IX. (from soil).—Marked, surface growth; spherical colonies, 4 to 12 mm. in diameter, dull ground glass appearance. Colonies ultimately assume crusted white appearance and show fine radial markings. × 50, present a characteristic frosted glass appearance, coarsely granular in centre with clear marginal zone (fig. 8).

Organism, non-motile, straight and curved rods, rounded ends, tendency to long thread formation; L. 4 to 8 μ, Br. 1 μ.

Gram :—Positive ; very small and terminal oval spore.

Bacillus X. (from soil).—Dull white, circular colonies, about 2 to 8 m. in diameter. × 50, are characteristic, yellowish colour, and show two zones—an inner finely granular about three-fourths of colony, and an outer zone with deeply indented margin showing delicate lace-like appearance and fluting on surface (fig. 9).

Organism, motile, numerous scattered rods, occasional chains, ends rounded; L. 6 to 10 μ, Br. 1·5 to 2 μ.

Gram :—Positive ; large terminal and oval spore.

Bacillus XI. (from soil).—Round, dull white colonies with moist, glistening appearance. × 50, appear yellowish brown, spherical, smooth contour, contents finely granular and freckled with faint markings, which, by reflected light, appear as a delicate reticulum.

Organism, non-motile, rods form long anthrax-like chains, straight and curved, ends rounded, about size of Hay bacillus.

Gram :—Positive ; terminal and oval spore.

Bacillus XII. (from soil).—Stellate, yellowish white colonies. × 50, appear yellowish white, and margin somewhat resembles an anthrax colony on agar at blood heat (fig. 10).

Organism, non-motile, forms long chains, ends somewhat flattened ; L. 4 to 8 μ, Br. 1 to 2 μ.

Gram :—Negative ; a drum-stick spore.

Bacillus XIII. (from soil).—Round, yellowish white colonies. × 50, appear spherical, brownish, finely granular with smooth contour, not very characteristic (fig. 11).

Organism, non-motile, rods plump, square ends, form chains; L. 4 to 8 μ, Br. 1·5 to 2 μ.

Gram :—Positive ; spore towards swollen end of bacillus.

Bacillus XIV. (from soil).—Thin surface film over agar, discrete and circular colonies at margin. × 50, appear granular, smooth contour, somewhat resemble a ball of cotton wool (fig. 12).

Organism, motile, short slender rods with rounded ends, surrounded by a zooglœa, no chains ; L. 4 μ, Br. 1 μ.

Gram:—Positive; oval spore towards centre of rod, giving a "fish-float" appearance.

The following table (I.) gives the appearances of the growths of the organisms in the ordinary culture soils of the laboratory, and is a further indication of the existence of a number of individual species of thermophilic bacteria.

The following are the main results of the above experiments:—There is in the first instance the good growth of all the above forms at 55°-65° C. on the ordinary culture media. It may be mentioned that their growth was likewise tested in sterile urine at thermophilic temperatures. In two instances no growth occurred in this soil. Of the others, a few gave a cloudy growth with general turbidity of the urine, whilst in other instances the urine remained clear, the growth appearing as a flocculent deposit in the test tube.

The organisms were all sporing forms of bacilli, and we did not succeed in isolating other forms of micro-organisms. Dr. Kidzior[6] has, however, described a thermophilic cladothrix as present in sewage and in the river Spree. This organism grew within sixteen hours in broth at 60° C. and showed genuine branching filaments. The organism is facultative anaerobic, and its temperature optimum is 55° C. It appears to produce a resistant spore or "dauerform," from which the threads develop at suitable temperatures. The observations of Rabinowitsch and others agree with our own as regards the isolation of exclusively bacillary forms of a sporing character.

The colonies presented distinctive appearances, especially those developing on salt potato agar. The growths in most instances were remarkably rapid; in one case a growth occurred over the whole surface of the agar within *six* hours. As a rule, good colonies were obtained after incubation for one night at thermophilic temperatures.

The majority of the bacilli showed a tendency to grow in long chains, and whilst branching-like forms appeared, we did not detect any genuine dichotomy.

Two organisms from soil (Bacillus X. and Bacillus XIV.) were the only bacilli that possessed undoubted motility

when observed in young cultures. Gram staining gave in some instances positive, in others negative results. It will be seen from the table that a number of these organisms do not grow on potato; in the positive cases the pigment production was striking and characteristic. The great majority curdled milk, and in seven instances liquefaction of the gelatine was produced. The indol reaction was positive in most instances, and only in one instance was it obtained without the previous addition of a nitrite to the broth. The growths on ordinary agar were not very characteristic.

The ubiquitous character of these organisms has been confirmed by other observers. Eight species are described by Rabinowitsch[2] as occurring in soil, water, the large and small intestine, &c., and in one instance in germinating barley at 62° C. The spores resist steaming for five to six hours. Dr. Oprescu[7] likewise describes five forms of thermophilic bacteria, which he classifies as follows:—(1) bacillus thermophilus aerobius (sewage water); (2) bacillus thermophilus aquatilis (Spree water); (3) bacillus thermophilus liquefaciens aerobius (soil); (4) bacillus thermophilus reducens (unsterile serum); (5) bacillus thermophilus liquefaciens tyrogenus (Roquefort cheese). A few isolated observations are likewise recorded by others.

Temperature Conditions.

We now proceed to a series of experiments made with the view of determining the range of temperature for the growth of thermophilic organisms. The experiments were made with pure cultures on agar, plain potato and potato agar. The results are given in the accompanying table (II.) The series at 22° C. are not included, as in each instance no growth was obtained at this temperature. It may be said that the growths were slower at the lower temperatures, occurring after one, two and three days, and were quicker at the higher temperatures, occurring usually within twenty-four hours. The range of temperature for each organism will be seen by running the eye across the table, which also

TABLE I.

The Growth of Thermophilic Bacteria in Culture Media.

No. of Bacillus.	Source.	Gelatin.	Agar.	Broth.	Potato.	Milk.
Bacillus I.	Animal dejecta	Copious flocculent deposit. Liquefaction, 14th day	Diffuse growth; not characteristic	Ropy viscous deposit; supernatant broth clear. Indol reaction negative	No growth	No action.
,, II.	Animal dejecta	Flocculent growth throughout gelatin; marked surface pellicle	Diffuse delicate surface film	Delicate surface pellicle and deposit; broth otherwise clear. Indol reaction positive	No growth	Viscosity, but no separation of casein.
,, III.	Soil	Ropy growth; no pellicle. Liquefaction, 5th day	Discrete, moist yellowish-white colonies, with smooth margins	Distinct stringy pellicle and viscous deposit. Indol reaction positive	Cream-coloured growth, of brownish tinge in old cultures	Solid curd, no whey.
,, IV.	Ensilage	Copious flocculent deposit; no pellicle	Dull white slimy growth over whole surface	General turbidity; no pellicle. Indol reaction positive	No growth	Solid curd, no whey.
,, V.	Thames mud	General turbidity and crusty pellicle	Diffuse dry film	General turbidity; surface pellicle. Indol reaction positive	No growth	Solid curd, no whey.
,, VI.	Plymouth sea water	Practically no growth	Diffuse transparent film	Faint growth. Indol reaction negative	No growth	Precipitation of casein and separation of whey.

,,	VII.	Soil	Flocculent growth throughout gelatin; no pellicle. Liquefaction, 4th day	Dry [illegible] growth	[illegible]	brick-dust-coloured growth	whey.
,,	VIII.	Soil	Fine flocculi throughout gelatin; copious deposit	Pale lemon-coloured growth of cream-like consistence	Faint general turbidity. Indol reaction positive	Distinct moist cream-coloured growth	Precipitation of casein and separation of whey.
,,	IX.	Soil	Gelatin remains clear; smooth surface pellicle	Diffuse growth, granular appearance; crusty and white in old cultures	Flocculent deposit; broth clear, no pellicle. Indol reaction positive	Snow-white dry crusty growth	Precipitation of casein and separation of whey.
,,	X.	Soil	General turbidity and deposit. Liquefaction, 3rd day	Dry enamel-like palmate growth	General turbidity. Indol reaction negative	Dry, felt-like reddish-brown growth	Solid curd, no whey.
,,	XI.	Soil	Stringy growth, mainly as deposit, which breaks up on shaking. Liquefaction, 7th day	Diffuse yellowish-white growth	General turbidity; slight deposit. Indol reaction positive	Moist brownish-yellow growth	Precipitation of casein and separation of whey.
,,	XII.	Soil	General turbidity; finely divided deposit. Liquefaction, 4th day	Diffuse dry growth	General turbidity and deposit; fine pellicle. Indol reaction positive	Dry pipe-clay-coloured growth	Solid curd, no whey.
,,	XIII.	Soil	Slight deposit. Liquefaction, 10th day	Dull yellow discrete colonies, with wavy margin	Flocculent growth. Indol reaction positive (without nitrite)	Moist yellowish-brown growth	Precipitation of casein and separation of whey.
,,	XIV.	Soil	Diffuse flocculent growth	Rapid diffuse growth	General turbidity and deposit. Indol reaction positive	Moist slate-coloured growth	Precipitation of casein and separation of whey.

TABLE II.

Illustrating Range of Temperature for Growth of Thermophilic Bacteria.

Temperature = Soil	37° C.			40° C.			42·5° C.			45° C.			46-47° C.			51° C.			55° C.			60° C.		
Organism.	A.	PA.	P.	A.	PA.	P.	A.	PA.	P.	A.	PA.	P.	A.	PA.	P.	A.	PA.	P.	A.	PA.	P.	A.	PA.	P.
Bacillus I.	M.G.	G.	O	M.G.	G.	O	M.G.	G.	O	V.G.	O	O	V.G.	V.G.	O	V.G.	V.G.	O	V.G.	V.G.	O	V.G.	V.G.	O
,, II.	O	O	O	O	O	O	G.	M.G.	O	G.	V.G.	O	V.G.	V.G.	O	V.G.	V.G.	O	V.G.	V.G.	O	V.G.	V.G.	O
,, III.	O	O	O	O	O	O	G.	G.	G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.
,, IV.	G.	M.G.	O	V.G.	V.G.	O	M.G.	G.	O	V.G.	M.G.	O	V.G.	V.G.	O	V.G.	V.G.	O	V.G.	V.G.	O	V.G.	V.G.	O
,, V.	G.	M.G.	O	M.G.	G.	O	M.G.	M.G.	O	V.G.	V.G.	O	V.G.	V.G.	O	V.G.	V.G.	O	V.G.	V.G.	O	V.G.	V.G.	O
,, VI.	O	O	O	O	O	O	G.	G.	O	G.	G.	O	V.G.	V.G.	O	V.G.	V.G.	O	V.G.	V.G.	O	V.G.	V.G.	O
,, VII.	O	O	O	G.	O	O	M.G.	M.G.	O	M.G.	G.	?	M.G.	V.G.	V.G.	V.G.	V.G.	?	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.
,, VIII.	G.	O	M.G.	M.G.	O	M.G.	M.G.	M.G.	M.G.	V.G.	M.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	G.	G.	V.G.
,, IX.	O	O	O	M.G.	O	O	M.G.	M.G.	O	V.G.	V.G.	O	V.G.	G.	G.	V.G.	?	G.	V.G.	V.G.	G.	V.G.	V.G.	G.
,, X.	O	O	O	O	O	O	G.	O	O	G.	O	O	G.	G.	G.	G.	?	G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.
,, XI.	O	G.	O	O	G.	O	O	G.	O	O	G.	O	G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.
,, XII.	O	O	O	O	O	O	G.	O	O	G.	O	O	G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.
,, XIII.	O	O	O	O	O	O	G.	O	O	V.G.	O	O	V.G.	V.G.	O	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.
,, XIV.	O	O	O	M.G.	M.G.	G.	V.G.	V.G.	O	V.G.	V.G.	G.	V.G.	V.G.	?	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.

Temperature = Soil	65° C.			67° C.			68° C.			69° C.			70° C.			72·5° C.			74·5° C.		
Organism.	A.	PA.	P.	A.	PA.	P.	A.	PA.	P.	A.	PA.	P.	A.	PA.	P.	A.	PA.	P.	A.	PA.	P.
Bacillus I.	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
,, II.	V.G.	V.G.	O	V.G.	V.G.	O	G.	G.	O	O	O	G	O	O	O	O	O	O	O	O	O
,, III.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	O	G.	G.	O	O
,, IV.	V.G.	V.G.	O	V.G.	V.G.	O	O	G.	O	O	O	O	O	O	O	O	O	O	O	O	O
,, V.	V.G.	V.G.	O	V.G.	V.G.	O	?	V.G.	O	G.	G.	O	G.	G.	O	O	O	O	O	O	O
,, VI.	V.G.	V.G.	O	?	?	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
,, VII.	V.G.	V.G.	G.	V.G.	V.G.	G.	V.G.	V.G.	G.	V.G.	V.G.	G.	V.G.	V.G.	G.	G.	V.G.	G.	G.	G.	G.
,, VIII.	O	O	V.G.	G.	G.	G.	O	O	G.	O	O	G.	O	O	O	O	O	O	O	O	O
,, IX.	V.G.	V.G.	V.G.	O	V.G.	V.G.	O	O	V.G.	O	O	O	O	O	O	O	O	O	O	O	O
,, X.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	G.	G.	V.G.	G.	G.	O	O
,, XI.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	O	G.	G.	O	G.	?
,, XII.	V.G.	V.G.	G.	V.G.	V.G.	V.G.	G.	G.	V.G.	G.	O	G.	O	O	O	O	O	O	O	O	O
,, XIII.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	V.G.	G.	V.G.	G.	G.	O	O
,, XIV.	V.G.	V.G.	?	V.G.	V.G.	O	O	V.G.	O	O	G.	O	O	G.	O	O	O	O	O	O	O

indicates the relative vigour of growth at the various temperatures.

It will be seen that all the organisms described grew best at thermophilic temperatures. On some of the soils a slow and moderate growth took place, in a few instances at 37° to 40° C., and still more gave evidence of growth at 42° to 47° C. We may, however, judging by our observations take 55° to 65° C. and even to 67° C. as an optimum temperature, both as regards the quickness and the amount of growth that occurred. It was interesting to note that in one or two instances a very good growth occurred at 72·5° C., whilst in a few cases evidence of growth was obtained even at 74·5° C. Another interesting point was the remarkable range of temperature at which growth could occur. Thus the Bacillus VIII. showed evidence of growth from 37° C. up to 69° C., whilst Bacillus XI. developed from 37° C. up to 74·5° C.—a remarkable range. At the same time there was no doubt about the optimum temperature being above 50° C.

The suggestion has been made that the phenomenon is simply an instance of ordinary saphrophytes adapting themselves to an abnormal temperature. It is difficult to believe that this is the case. Such adaptiveness or "anpassung" requires time and a careful training of the organism under ordinary circumstances. The growths were too good and rapid to lend support to such a theory. However they may live under ordinary circumstances, there is no doubt with regard to the favouring influence of the factor, temperature. At the same time the varied conditions that must present themselves in nature may favour their growth, even if it is slow, *e.g.*, certain chemical conditions, not represented by the culture soils of the laboratory, of which we are at present ignorant.

The suggestion that it is a question of adaptivity on the part of ordinary bacteria led to a series of experiments with some well-known forms—we had already proved that the ordinary saprophytes will not grow at the optimum thermophilic temperature.

The following short table (III.) gives the results obtained.

The conclusion arrived at was, that as regards conditions of temperature the mesentericus group of organisms appears

TABLE III.

Temperature range of typical saprophytic organisms.

Organism.	Temp. 37° C.	Temp. 40° C.	Temp. 42·5° C.	Temp. 45° C.	Temp. 46-47° C.	Temp. 50° C.
B. mesentericus vulgatus ..	+	+	+	+	+	+
,, ,, niger	+	+	+	+	+	+
,, vermicularis	+	+	+	+	+	+
,, mycoides	+	+ ?	O	O	O	O
,, ramosus	+	+ ?	O	O	O	O
Proteus vulgaris	+	+	+ ?	+ ?	O	O
S. pastorianus II.	+	+ ?	O	O	O	O
,, ellipsoides I.	+	+ ?	O	O	O	O

to form a connecting link between the ordinary saprophytic and the thermophilic bacteria.

As regards optimum temperature, the micro-organisms commonly fall into three great groups—

(1) For the great majority of saprophytic bacteria an optimum of 15° to 20° C.

(2) For the parasitic forms an optimum of 37° C.

(3) For the thermophilic organisms an optimum of 55° C. or thereabouts.

The facultative parasites, such as the bacillus anthracis, form a link between the first two groups, and grow well at the respective temperatures. The mesentericus group connect the second and third group.

Anaerobic Experiments.

The organisms isolated had hitherto been studied under aerobic conditions, which appeared in every way the most suitable for their growth. A series of experiments was made to discover whether the pure strains isolated could grow anaerobically, and also whether by direct inoculations with soil the existence of anaerobic forms could be proved.

The anaerobic cultures (hydrogen atmosphere) made directly with soil in broth gave an abundant growth of slender bacilli. The broth had a foul odour, and became blackened. Deep cultures of soil in potato agar gave a slow development of colonies. In one instance subcultures were made from the broth tubes in the depth of agar tubes, and the surface sealed with gelatine. Colonies appeared below the surface, and a certain amount of gas and a butyric odour were developed. All anaerobic inoculations of soil in broth at thermophilic temperatures gave a growth and offensive odour. There were therefore forms capable of growth under anaerobic conditions.

A number of pure cultures of the organisms were placed in gas jars containing an alkaline solution of pyrogallic acid, and a certain number in sealed Hueppe's tubes. These experiments did not succeed, and led to the conclusion that the thermophilic bacteria we had isolated require oxygen for their growth. All the tubes on being placed in free contact with air developed growths. Experiments were likewise made with deep cultures in the salt potato agar. No growth occurred along the line of the stab, but in each case a good surface growth occurred in contact with the air. Like the growths on potato, the surface growths on potato agar developed various pigments—yellow, brown, pink, &c.

The growths that do occur (*e.g.*, from soil), under anaerobic conditions require further investigation, and it is hoped to deal more exhaustively with this subject in a later paper on the cellulose fermentation.

Rabinowitsch states that the forms she isolated were facultative anaerobes, and that at low temperatures they frequently grow quicker anaerobically, especially in broth. This suggests the possibility of an anaerobic growth of these organisms in nature at ordinary temperatures.

To test these suggestions we made deep cultures in sugar agar, and kept them at blood-heat for about three months, but there was practically no deep growth and only a trace of surface growth in two instances. The tubes, however, on being placed at 55° C. immediately developed growths which were mainly on the surface.

"Shake" cultures in ordinary agar at 22° and 37° C. likewise gave negative results.

Broth cultures in hydrogen showed no growth after ten weeks at blood-heat. The tubes were then opened so as to admit air, and placed at 55° C., when a good and immediate growth occurred in every instance. Further experiments always gave the same results, whether carried out with fluid or solid media. The results were, therefore, negative as regards an anaerobic growth of the organisms at the lower temperatures. Rabinowitsch's results are not decisive, and do not appear to have been of a uniform character, as it is stated that at times no growth occurred for some unexplained reason. We do not think that this theory will explain their active existence in nature under the ordinary conditions of temperature. It is, however, but reasonable to suppose that such a widely distributed group of organisms must find favouring natural conditions outside the factor of temperature, these conditions being probably of a chemical nature. We are equally ignorant of the life conditions of many pathogenic organisms outside the body. At the same time, distinct evidence was obtained of the anaerobic existence of certain thermophilic forms at high temperatures.

Action of Light.

A series of cultures were exposed on the roof during sunny weather during a period of twelve days. There was some evidence of growth in two cases. On placing the tubes at 55° C. they remained sterile with two exceptions. Of the fourteen organisms twelve had been killed by the direct sunlight.

Variations in Form.

A number of experiments were made to test the effect of temperature in producing any possible variations in form of the organisms. Three typical bacilli were cultivated at gradually increasing temperatures, viz., from 40° C. up to 70° C. The results may be shortly summarised as follows:—At the lower temperatures bacilli in short or long chains

were generally obtained. At the thermophilic temperatures the tendency to thread formation became more marked, as well as a tendency to develop interlacing cladothrix-like filaments without, however, a genuine branching. The organisms always retained the character of bacilli at the various temperatures.

Action on Culture Media.

(1) *Reaction.*—The organisms were capable of growth in sugar broth and litmus broth.

In the first experiments made the sugar broth became acid at the end of six days, whilst a bleaching generally occurred in the simple litmus broth.

A sugar-free peptone beef broth was made from stale meat and carefully neutralised. The fourteen organisms were cultivated in this broth at 55° C. No acid was detected in the sugar-free broth and the tubes gave a more or less alkaline reaction. The tendency appeared to be in the direction of alkali formation.

In litmus agar the organisms produced a bleaching of the litmus in twenty-four to forty-eight hours with one doubtful exception.

In sugar agar a growth appeared in each instance, though more slowly than in a non-sugar soil. No gas development took place.

On sugar litmus agar after forty-eight hours Bacillus X. and Bacillus XII. showed a reddening of the litmus, the others showed slight alkalinity or no distinct change occurred.

(2) *Reducing properties.*—The nitrate solution used had the following composition:—Potass. phosph., 0·1 grm.; magnes. sulph., 0·02 grm.; calc. chlor., 0·01 grm.; potass. nit., 0·168 grm.; dextrose, 0·3 grm.; peptone, 0·25 grm.; aq. d. 1000 grm.; and calc. carb., 4 grms. This fluid was inoculated with pure cultures and incubated at 55° C. and finally tested with Nessler's reagent. The control tubes tested for nitrites and ammonia gave negative results, whilst tubes inoculated directly with soil gave a good reaction. As regards the pure cultures of the organisms, the results with

Bacilli I., IV. and V. were negative. Bacilli II., III., IV. and XI. gave very good reactions and fair reactions were given by the remaining organisms. The positive tubes on testing with a solution of sulphanilic acid gave the nitrite reaction, showing that a reduction had taken place. The above experiments were repeated with similar results, on testing with Nessler's reagent and sulphanilic acid. The majority of the organisms, therefore, possessed reducing properties.

(3) *Action on proteids.*—It was already noted that a certain number of the organisms produced a liquefaction of gelatin.

A series of tubes of inspissated *serum* were inoculated directly with soil and incubated at 55 to 65° C. A complete liquefaction of the serum was produced and a most offensive smell. The action of pure cultures of the organisms on serum were tested under the same conditions. The growths were slower than on the ordinary culture media.

At the end of four days eight of the bacilli gave a medium or good growth on the surface of the serum without any evidence of liquefaction. The remaining six bacilli gave good growths accompanied by a pitting or complete liquefaction of the serum, viz., Bacilli I., II. and XIII. produced pitting and Bacilli VII., IX. and XI. distinct liquefaction of the serum.

Flasks containing *meat* with sterilised humus and a small amount of peptone water were inoculated aerobically with the mixed growths obtained by sowing soil on blood-serum. The meat underwent an active decomposition through the action of the mixed growths.

Another series of flasks was inoculated with pure cultures of the bacteria, but, although a slight odour was evolved, no results were obtained to compare with those due to the mixed growths as contained in soil.

Blood albumen, inoculated directly with soil, gave vigorous growths, along with an active decomposition and a most repellent odour. The pure cultures, again, did not give results corresponding in intensity or completeness. In some instances no effect was produced; in other instances a slight

disintegration of the albumen took place, with a putrid, a mousey, or an aldehyde odour.

Eggs, inoculated with soil, developed a distinct smell, and their contents were blackened. With pure cultures a growth occurred in the eggs, but the natural mixture of organisms always gave better results.

It would, of course, be advisable, in this connection, to make anaerobic experiments as well, but there was no doubt as to the decomposition of proteid material by these organisms, especially in mixed cultures obtained directly from soil. The action appeared to be of a symbiotic nature.

(4) *Action on Carbohydrates.*—The organisms, in pure culture, were added to a 2 per cent. solution of *cane sugar* in peptone water at first, but finally the sugar solution was used alone, and was found to be preferable. The tubes were incubated at 55° C. Eight of the organisms were found to invert cane sugar, viz., Bacilli III., VI., VII., IX., X., XI., XIII., and XIV. The organisms were further grown on a *starchy* soil at 55° C. The starch was diastased by four organisms, viz., Bacilli IV., IX., X., and XIII. The organisms multiplied in the starch solution, which became yellow on adding potash and reduced Fehling's solution.

The thermophilic bacteria had, therefore, an inverting and diastatic action at these high temperatures. Dr. Oprescu found that two of the five bacilli he examined exhibited both a proteolytic and a diastatic action. The action of the proteolytic enzyme ceased at 60° to 65° C., but the diastatic was more resistent, as one hour at 65° C., and even one hour at 80° C., did not inhibit its action.

Experiments with Ensilage.

Generally speaking, ensilage is a process for the preservation of green fodder, in which the fodder is so packed that, by dryness and the absence of air, the acetic acid fermentation is hindered, and the lactic acid fermentation is favoured. As is well-known, the "silos" attain a considerable temperature. It was therefore of interest, in this connection, to examine samples of the silos. The first samples were

obtained from Devonshire; the temperature inside the stack was not known, but the edge was 78° F. at the time of removal. Samples of the straw at 60° C. gave growths in every instance, and plate cultures were made from these mixed growths. It was noticed that in the original tubes a second series of growths had started, of a whitish colour, on the straw itself. These growths appeared more slowly, and appeared to prefer the straw to the agar as a soil. We noticed growths of similar appearance in the course of the decomposition of cellulose to be referred to subsequently.

A second sample from a stack (temperature 50° C.) yielded quick and slow growths, which appeared after six days. Microscopically, a large number of bacilli were observed.

A sample of ensilage was placed in a tin box, and hermetically sealed, and incubated at 60° to 65° C. When opened at the end of five weeks it was found to be still moist and steaming, and to be covered, in parts, with a whitish growth. The whole of the ensilage was swarming with living bacilli; and numerous spores were present. Cultures were made at 22° C. and 37° C., with negative results. With cultures kept at 60° C., a copious growth was obtained within eighteen hours. The ordinary saprophytes had been killed, and only the thermophilic bacteria had remained alive and active. The fodder kept in the box at 60° C. for two months gave copious growths, at the end of that time, of thermophilic bacteria, and no growths, either aerobic or anaerobic, occurred at 22° C. and 37° C. A hanging drop showed many bacilli, including motile forms. It was most interesting to find that after two months at 60° C., the ensilage was swarming with active bacteria. There could not be a more conclusive experiment as to the favouring conditions of a high temperature on these forms of life.

Experiments with Cellulose.

The occurrence of thermophilic bacteria in ensilage, and their relation to the spontaneous heating of hayricks led us to consider that these organisms probably exerted some action on cellulose. The first experiment made was quite

unexpectedly successful. To a solution of nutrient salts containing a trace of broth, were added Swedish filter paper and a small quantity of soil. The mixture was incubated at at 60° C. The result was an active development of gas and smell. In about ten to fourteen days the filter paper was completely broken up. A microscopical examination on the fourteenth day showed numerous bacilli, some of which were sporing. There were also small bacteria present embedded in a zooglœa—an undoubted disintegration of the cellulose had occurred. We repeated these experiments with filter paper, and also with samples of pure cellulose, for which we are indebted to Mr. Cross. A series of tubes, after inoculation with soil, were incubated at 22° to 37° and 60° C. In one series the tubes were aerobic, in another anaerobic. The fermentation of the cellulose started slowly and first became marked about the fourteenth day. A complete decomposition of the cellulose occurred at the end of three weeks at 60° C., whilst at 22° and 37° C. no action was evident. The results were so striking that it was felt advisable to have a control analysis made, and this was very kindly carried out by Mr. Cross. The cellulose used consisted of prepared films of cellulose hydrate, as obtained from the solution of the original fibrous cellulose (cotton) in the form of thiocarbonate. The samples represented a chemically pure cellulose of the normal group in its most reactive condition (Cross). We also obtained from Mr. Cross specimens of pure straw and esparto cellulose.

Mr. Cross reported as follows on the solutions, which showed a disintegration of cellulose by the thermophilic organisms :—

"(1) No reduction CuO, in original, nor after boiling with acid.

"(2) No other evidence of any proximate products of resolution, *i.e.*, carbohydrates of dimensions, $N.C^6$.

"(3) On distillation, 25 c.c. gave volatile acid = 1 c.c. of normal NaOH, containing acetic and butyric acids. Residue gave traces only of furfurol on distillation with HCl (1·06 s.g.).

"It appears that the destruction has been for the most

part complete, probably to CO_2 and CH_4. On further investigation you may be able to get an intermediate stage or an organism acting less severely."

These experiments therefore gave undoubted evidence of the digestion of cellulose by thermophilic bacteria.

The experiments were continued in the following manner:—A series of flasks was prepared containing pure cellulose and Naegeli's nutrient solution + 5 c.c. of nutrient broth, and a second series containing broth alone and cellulose. These were inoculated with soil and kept at 60° C. After about three days an active fermentation started in the flasks of cellulose and broth, but had not yet appeared in the flasks without broth. The traces of broth appeared to help the fermentative process.

Anaerobic experiments were also made in Hueppe's tubes containing Naegeli's solution, broth, pure cellulose and a small quantity of soil. After nine to ten days the cellulose was attacked in the anaerobic, as well as in a series of aerobic tubes, but appeared to be more advanced in the anaerobic tubes. A marked smell occurred in both instances. A large number of bacilli were present, and we specially noted that several new forms appeared that had not been noted in the experiments without cellulose.

A further series of experiments with well washed esparto cellulose resulted in the disappearance of the cellulose at the end of three weeks at 60° C. In this instance a mixed culture from fermented cellulose had been added. The control tubes kept at 22° to 37° C. were not visibly affected after four weeks.

The rate of fermentation and disintegration of the cellulose varied from seven to twenty-one days as a general rule.

All the above results were brought about by mixtures of thermophilic bacteria, occurring naturally in the soil and the action appeared to be of a symbiotic nature. Their action resulted in a *complete disintegration* of filter paper, fibrous cellulose and esparto cellulose.

The further discussion of the fermentation of cellulose must be left for a subsequent paper.

So far, these experiments, whether with proteids or cellulose, always gave the best results when a mixture of organisms was present. It may also be that the addition of soil supplied valuable constituents for their growth, and that decoctions from soil may prove of help in the future study of these organisms.

General considerations.

Without considering any possible chemical or other favouring conditions that may exist, in how far can such organisms find in nature the temperatures that are so well suited for their growth? It was suggested in our first paper that in the course of many natural fermentations the heat developed frequently reached a temperature suitable for thermophilic bacteria; we have since then looked into the subject, and obtained some direct evidence on the point.

The following are readings taken in a *silo* :—August 22, 120° F.; 23rd, 150° F.; 24th, 140° F.; 25th, 130° F.; 26th, 160° F.; 27th, 160° F; 28th, 150° F. The silo remained at 150° F. for two months.

A pit of stable manure gave following readings:—At 1 ft., 60° C.; 2 ft., 65° C.; 3 ft., 62° C.; 6 ft., 70° C.; and a heap of fresh manure after twelve hours rose to 50° C.

A heap of field manure at the surface was 40° C.; 1 ft. deep, 60° C.; 3 to 4 ft. deep, 70° C.

Refuse moss litter gave temperature of 42° to 60° C.; and straw litter 51°, 75°, 83° and 84° C. A small heap of hay saturated with urine and fæces had a maximum temperature of 71° C.

The temperature of a heap of straw litter during six days is given in the following table :—

	1st day.	2nd day.	3rd day.	4th day.	5th day.	6th day.
2 feet	63° C.	60° C.	60° C.	60° C.	63° C.	50° C.
3 „	78° C.	75° C.	70° C.	71° C.	75° C.	72° C.
5½ „	82° C.	85° C.	83° C.	81° C.	84° C.	87° C.

These temperatures are, of course, mostly favourable for the development of thermophilic organisms.

The following temperatures have been noted in other fermentations, associated with bacteria:—

Spontaneous heating of hay and cotton	67° C.
Germinating barley	60° C.
Tobacco fermentation	54° C.
Snuff ,, ...	50° C.
Air-dried hay	70° C.
Brown hay	70° C.
Lactic acid fermentation	52° C.
Butyric acid fermentation	40° C.

The spontaneous heating of hops is associated with an organism, the bacillus lupuliperdi. There is a wide and profitable field of research open with regard to the fermentations that occur at 50° to 60° C. In the spontaneous heating of hay ricks the temperature rises to 50° to 60° C. through the agency of bacteria, and chemical processes are thereby started which lead to the production of further heat and inflammable bodies.

The enormous amount of manure that is constantly undergoing decomposition must furnish a most favourable nidus for thermophilic organisms, and we are sometimes apt to forget the amount of heat that is continually being developed at all seasons of the year by the processes of nature.

The forms of life we have been studying constitute a large and important class of organisms endowed with a marked vital activity, which finds its most active expression at thermophilic temperatures, as is evidenced by the fermentative and other changes they are capable of bringing about under such conditions. We have a most striking example of this in the complete disintegration of cellulose under their influence. The exact conditions that may probably favour their growth at subthermophilic temperatures are not yet known—they may possibly be of a chemical nature.

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FIG. 1

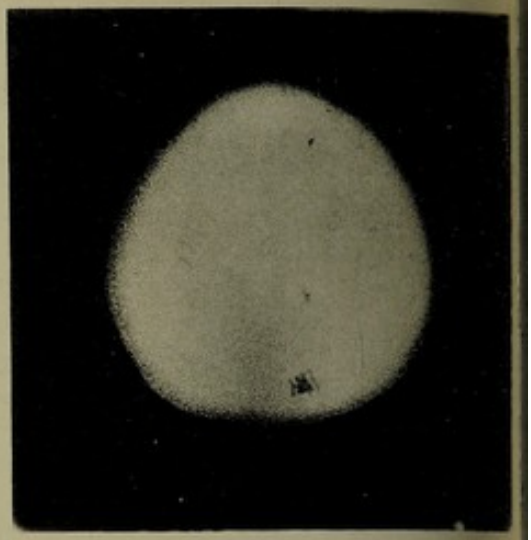

FIG. 2

FIG. 3

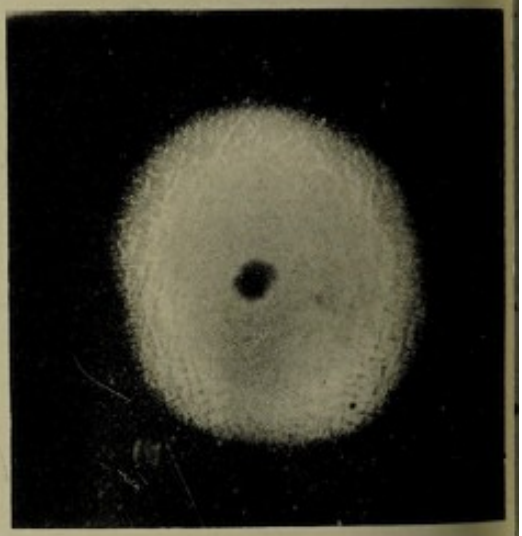

FIG. 4

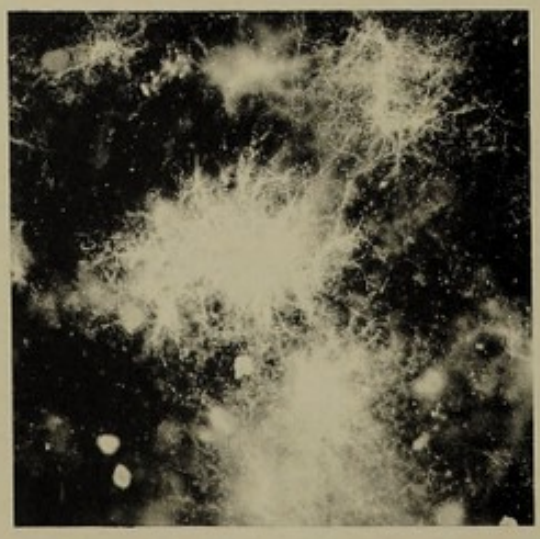

FIG. 5

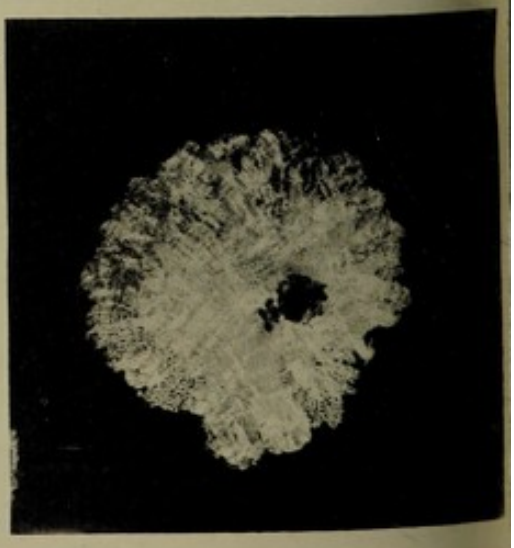

FIG. 6

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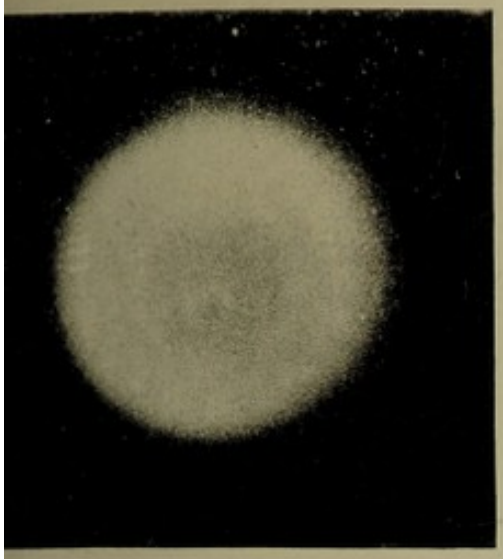

FIG. 7

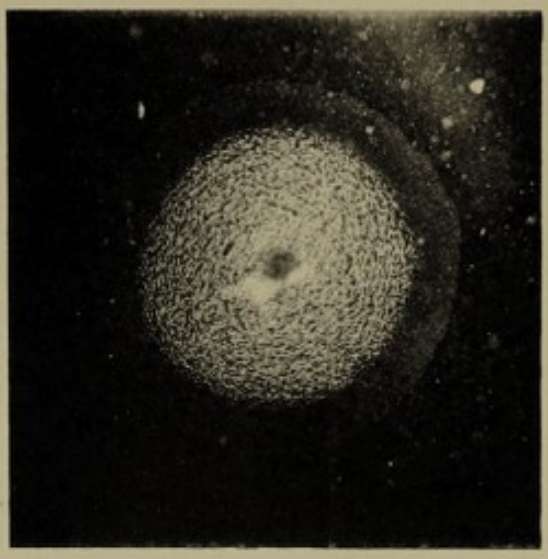

FIG. 8

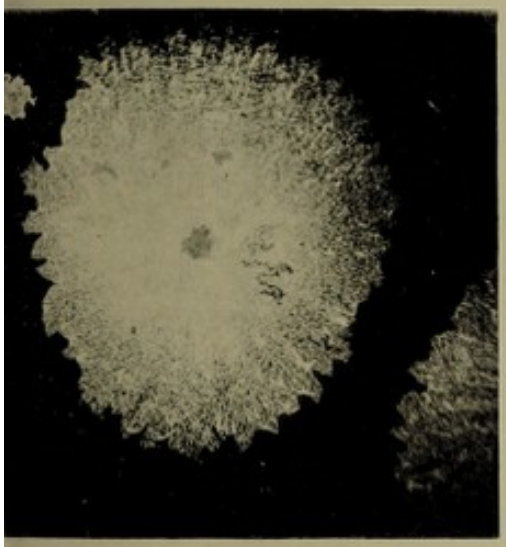

FIG. 9

FIG. 10

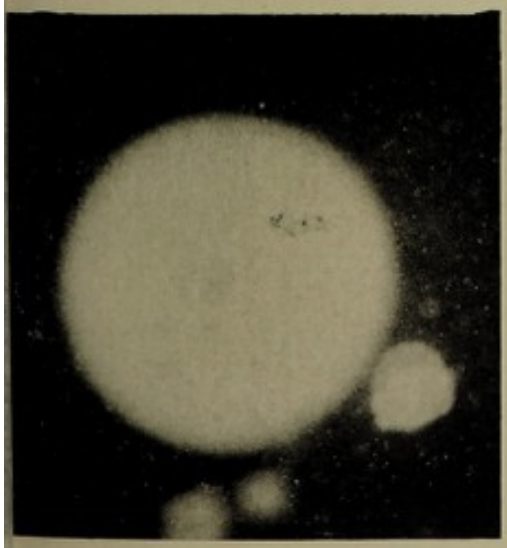

FIG. 11

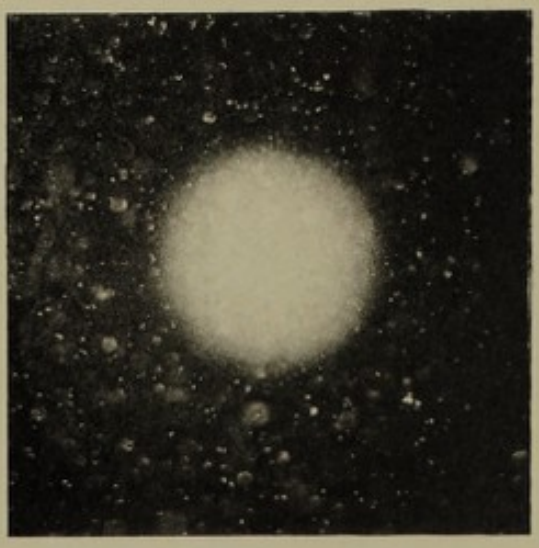

FIG. 12

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FIG. 13

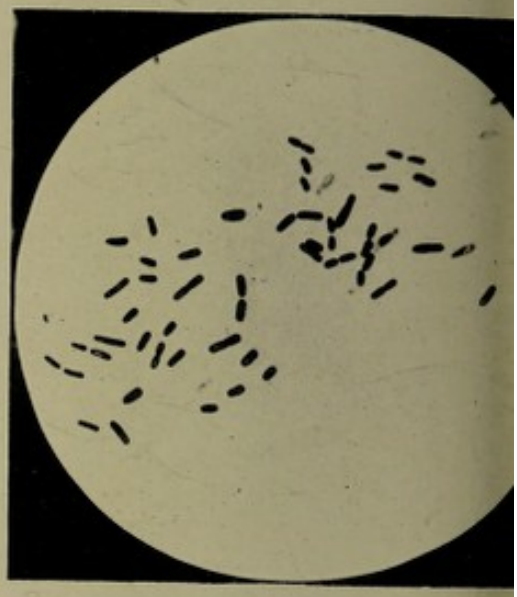

FIG. 14

FIG. 15

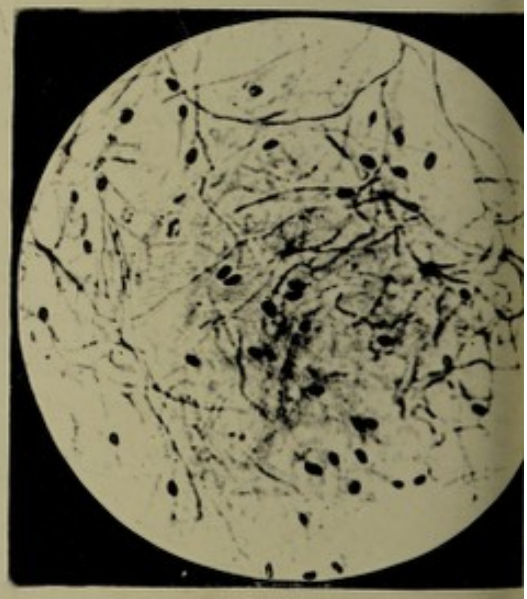

FIG. 16

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DESCRIPTION OF PLATES.

Fig.	1.	Bacillus	I.	(from dejecta) .. Plate colony.
,,	2.	,,	II.	(,, ,,) .. ,, ,,
,,	3.	,,	IV.	(,, ensilage) .. ,, ,,
,,	4.	,,	V.	(,, Thames mud) ,, ,,
,,	5.	,,	VI.	(,, Plymouth seawater) ,, ,,
,,	6.	,,	VII.	(,, soil) .. ,, ,,
,,	7.	,,	VIII.	(,, ,,) .. ,, ,,
,,	8.	,,	IX.	(,, ,,) .. ,, ,,
,,	9.	,,	X.	(,, ,,) .. ,, ,,
,,	10.	,,	XII.	(,, ,,) .. ,, ,,
,,	11.	,,	XIII.	(,, ,,) .. ,, ,,
,,	12.	,,	XIV.	(,, ,,) .. ,, ,,
,,	13.	Thermophilic bacillus.		Growth at 55° C.
,,	14.	,,	,,	,, ,, 65° C.
,,	15.	,,	,,	Sporing Stage.
,,	16.	,,	,,	Cladothrix-like growth, at maximum temperature; numerous spores.

The plate colonies and cultural peculiarities present the best means of differentiating the above forms.