IX. *Contributions to the Chemical Bacteriology of Sewage.*

By Sir Henry E. Roscoe, *F.R.S., D.C.L., LL.D., and* Joseph Lunt, *B.Sc., F.C.S., Associate of the Owens College.*

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[Plates 40–43.]

The present paper contains the results of the chemical and bacteriological examination of sewage, with the object, in the first place, of ascertaining the species of organisms therein contained, and, in the second, of determining some of their chemical characteristics.

Of late years attention has chiefly been paid to the study of pathogenic organisms, whilst the far more numerous class of saprophytic organisms, which, although not associated with disease, nevertheless play a most important part in nature, has only been examined by a few investigators.

The great progress which has recently been made in our knowledge of pathogenic processes from a bacteriological point of view is, to a great extent, due to the method of plate culture on solid media, proposed by Koch, whereby it is easy to obtain pure cultures, so that the study of the life-history and functions of the innumerable micro-organisms occurring in nature thus becomes possible.

The authors have isolated from crude sewage, by methods afterwards described, a number of organisms which may serve as typical examples of those usually present in sewage. Some of these have already been described, whilst others are believed to be new organisms. All the organisms described in this paper were isolated from the crude sewage flowing into the sewage works at Acton, London.

The microscopic and macroscopic appearances of the organisms and their pure cultures have been carefully recorded by means of photographs, which give in a permanent form their morphological characters, and the appearance of the plate and tube cultivations in their most characteristic stages of growth. This method of exact illustration the authors consider to be of much importance, as bacteriological descriptions of organisms are frequently of little value owing to the absence of accurate representations of the microscopic preparations and pure cultures.

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The experiments hereinafter described were undertaken with the object of studying the reactions of sewage organisms from a chemical point of view, and of gaining information as to the *rationale*, both chemical and bacteriological, of the two marked changes which sewage is liable to undergo, *i.e.*, on the one hand, purification, or the gradual destruction of putrescible matter without the formation of offensively smelling products, and, on the other hand, putrefaction.

For this purpose it was necessary to determine which of the organisms are concerned in the first of these processes, and which in the second, as likewise to gain an insight into the methods by which such changes are effected, for this is little known.

PASTEUR, nearly 30 years ago, in his classical memoir on spontaneous generation, proved that certain forms of microbic life possess the power of rapidly absorbing free oxygen from the atmosphere ('Ann. Chim. Phys.,' vol. 64, 1862, p. 78), but as the present methods of obtaining pure cultivations of such organisms were then unknown, he was unable to study the effect of each organism separately as regards the absorption of oxygen.

For all the organisms described in this paper, the authors have determined the absorptive power for free oxygen, when cultivated in a perfectly pure state, and also to which of the organisms free oxygen is a necessity of their activity and growth.

PASTEUR again, in 1863, first showed that certain micro-organisms could carry on their life and growth in a nutrient liquid from which the air had been expelled by boiling ('Comptes Rendus,' vol. 56, 1863, p. 416), but it is very doubtful whether he worked with pure cultivations, as they were obtained spontaneously from the dust of the air.

As it is still a point upon which differences of opinion exist as to whether micro-organisms can grow and multiply in absence of the smallest trace of oxygen, each organism has been examined as to its power of growth in a liquid nutrient medium from which every trace of free oxygen, both gaseous and dissolved, has been rigorously excluded.

PASTEUR further pointed out that free access of air was unfavourable to putrefaction, and he believed that it was occasioned by the growth of anaërobic organisms, which were unfavourably affected by the presence of free oxygen, and that the way for their action was prepared by another set of microbes which were aërobic, and used up the oxygen, replacing it by carbonic acid gas. In this relation it will be shown that the anaërobic organisms, producing putrefaction, are themselves (in pure cultivations) capable of absorbing free oxygen with the production of carbonic acid gas, thus preparing the way for their further anaërobic growth.

Various other points of interest have appeared during the progress of the research, which will be more specifically referred to in their proper place without anticipating them here.

The following pages contain detailed descriptions of the experimental methods followed, after which the organisms found in sewage are severally described, with

photographic illustrations, and conclude with a short *résumé* of results and an appendix containing tables and minor details of methods used.

Description of Experimental Methods Followed.

The following methods for the isolation of organisms have been used:—

(1) The method of gelatine plate culture.
(2) A method, to be described later, for the isolation and cultivation of anaërobic organisms.
(3) A method for the isolation of spore-forming organisms.
(4) The dilution method.

All the cultivations, unless otherwise stated, were made at a temperature of 20° to 23° C. as nearly as possible.

Plate Cultivations and the Preparation of sterile Gelatine Tubes.

The exact method followed, differing somewhat from the general practice, will be found described in the appendix.

Method for the Isolation of Anaërobic Organisms.

The question arose whether the usual method of plate cultivation, carried out, as it is, in contact with air, was equally adapted for the cultivation of anaërobic as well as for aërobic organisms, and it was necessary, in the first place, to ascertain whether any anaërobic organisms existed in the sewage under examination. For this purpose a special form of cultivation flask was devised (fig. 1), suitable not only for mixed cultures, but also for pure cultures, in which the organisms can be grown in an atmosphere of pure hydrogen in absence of every trace of free oxygen.

The flask is furnished with a capillary tube *e*, sealed in at *f*, for the purpose of introducing hydrogen. Foreign germs are excluded by a firm plug of sterile wool at *g*.

When it is required to sterilise the flask and its contents before the introduction of pure material or sewage, the fine jet *a* is sealed, and the opening *c*, for the introduction of the culture fluid and organisms, is protected by a sterile plug *d*. The whole is now steamed for 20 minutes on two or three successive days, and is then ready for use.

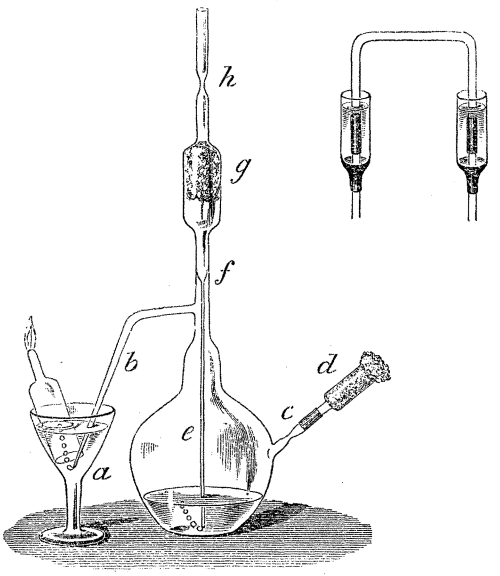
The plug *d* is carefully removed, and a few drops of sewage are introduced by a freshly drawn out capillary pipette, after which the tube is sealed at *c*.

Pure hydrogen is now passed through the liquid (nutrient peptone broth described in the Appendix) by means of the capillary tube *e*, the gas issuing by the broken off end of tube *a*, immersed in water to shut off all communication with the air. The gas is furnished continuously by an apparatus described by the authors in the 'Chemical Society's Transactions' for 1889, p. 561.

After the gas has passed for half an hour, and every trace of oxygen above the

liquid as well as that dissolved* in it is expelled, the flask is hermetically sealed at *h* and *b*. To prevent internal pressure blowing out the glass on sealing, a little mercury is run into the conical glass until *a* is covered, then a tap in the hydrogen apparatus is opened so as to reduce the pressure, after which the sealing can be accomplished in safety.

Fig. 1.



Hydrogen Flask for Anaërobic cultures.

With the object of eliminating all organisms which could not grow in absence of oxygen, a few drops of the turbid and putrescent broth from the first hydrogen flask, after five days' incubation, were sown in a second flask which was filled with hydrogen as before described, and a little material from this second flask was sown in a third similar flask.

By this treatment, not only were all aërobic organisms eliminated, but on making plate cultures of the broth in the usual way in air, one organism alone appeared, and this method may be adopted for its isolation. It is described on p. 644, and closely corresponds to *Proteus vulgaris*.

Method used for the Isolation of Spore-forming Organisms.

A few drops of sewage are introduced into a sterile broth tube by means of a recently drawn out capillary pipette, and the plug is replaced. The tube is now plunged into water at 80° C. for 10 minutes, which treatment kills all the full-grown bacilli, but is not sufficient to kill the spores. The spore-forming organisms may now be isolated by plate culture, either with or without previous incubation of the partially sterilised broth tube.

The Dilution Method.—This method may be found useful in isolating certain forms

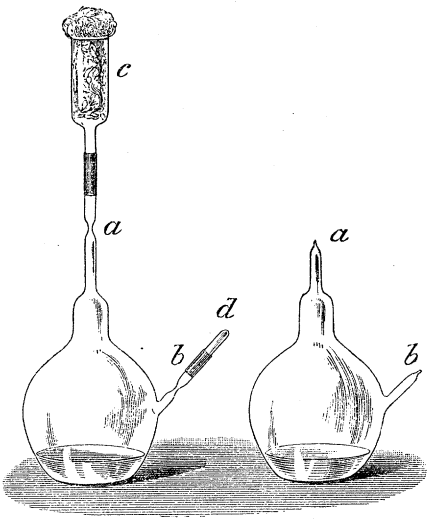
* The authors have proved this. (See 'Chem. Soc. Trans.,' 1889, p. 554.)

which are not readily separated by plate cultures alone. A few drops of sewage are introduced into a 50 cub. centim. Chamberland flask filled with sterile water. From this, after mixing, a drop is taken into a second flask of sterile water, and from this, again, into a third containing sterile broth. After a few days incubation the broth is examined microscopically. Usually not more than two distinct organisms appear, which are then easily separated by plate culture. In this manner a new organism (Anaërobic No. 3) was isolated, its extremely characteristic growth in broth pointing it out as a distinct species.

Reaction of Pure Cultures towards Oxygen.

For the purpose of studying the reaction towards atmospheric oxygen, the organisms were cultivated in the pure state in sealed flasks containing 25 cub. centims. of sterile broth and about 250 cub. centims. of air. A trace of a recent pure culture was used for sowing, and in each case the purity of the subsequent growth was tested by plate culture or microscopic observation.

Fig. 2.



Flasks for Estimation of Oxygen absorbed.

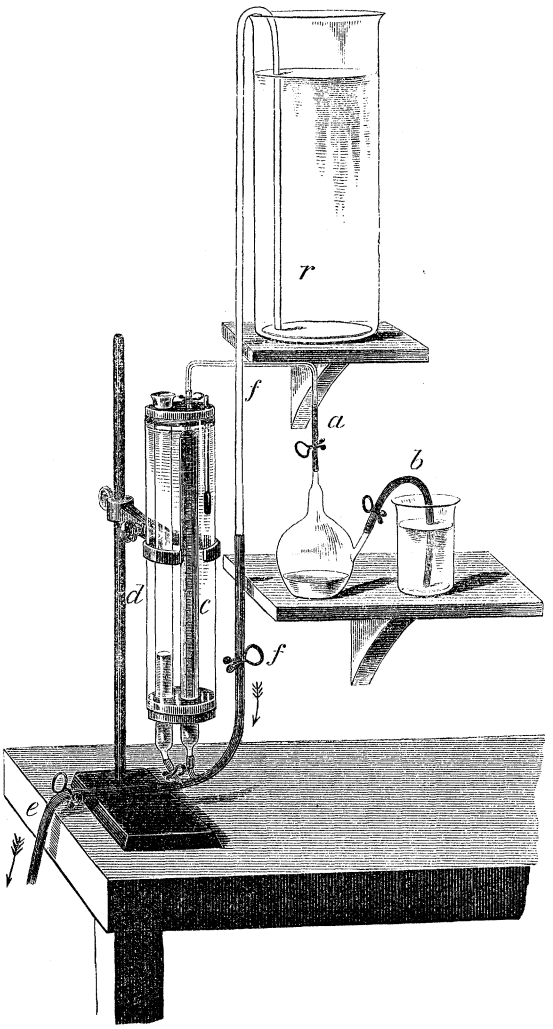
The flasks were furnished with two necks as shown in fig. 2. The lateral neck is plugged by a piece of glass rod *d*, during sterilisation, and after the organisms have been introduced by means of a sterile platinum wire, is sealed off at *b* which afterwards serves for the entrance of water, through the broken end, to replace the gas when it is abstracted for analysis.

The upper neck is connected with a sterile cotton-wool plug *c*, which serves to filter the air entering the flask on cooling after steam sterilisation. This neck when broken also serves for the exit of the gases for analysis, after the incubation of the organisms.

After sowing and sealing off at *a* and *b*, the flasks were incubated at 20°–23° C. for seven days, after which time they were connected by india-rubber tubing filled with

water (fig. 3, *a*) with a Hempel's gas burette and pressure tube surrounded by a water jacket to give a uniform temperature, and by *b* with a beaker of water used for replacing the gas abstracted for analysis.

Fig. 3.



Method of Estimating Oxygen absorbed.

On breaking the tubes at *a* and *b*, and opening the appropriate pinchcocks, the gas enters the gas-tube *c* previously filled with water, the water flowing out through *e*. It only now remains to connect the Hempel's gas absorption bulbs at *a* instead of the flask, and to force the gas over by water pressure conveyed through *f*.

The carbonic acid and oxygen were absorbed by caustic potash and phosphorus respectively.

This method of analysis is not relied on for scrupulous exactness, but the results obtained are sufficiently accurate for the purpose aimed at. The amount of

carbonic acid absorbed by the water was neglected, as also was the interchange of dissolved gases.

The results of the analyses of the gases are appended in Table I. of Appendix (p. 661).

It will be seen that the various organisms exhibit great differences in their absorptive power for free oxygen, some showing the feeblest absorption, whilst others abstracted nearly every trace of oxygen from an atmosphere ten times as large as the culture liquid, during seven days' incubation. For comparison the results are expressed in parts of oxygen remaining and CO_2 produced per 80 volumes of nitrogen, 20 volumes of oxygen representing pure air.

On the Rate of Absorption of Dissolved Oxygen by Pure Cultures.

For the purpose of studying the rate at which dissolved oxygen is absorbed from water by the various pure cultures experimented with, tap water, previously sterilised in the steam steriliser and allowed to cool, was fully aërated at the temperature of the incubator 21–22° C. as described in a previous paper by the authors ('Chem. Soc. Trans.,' 1889, p. 567). To a Winchester quart bottle of the standard aërated water was added 1 per cent. by volume of broth which had been sown with a minute trace of a pure culture two days previously, and incubated at 20–23° C.

Preliminary experiments with an organism (Aërobic No. 4) which absorbed oxygen rapidly showed that in three hours the action had only just commenced, whilst after twenty-one hours all but a trace of oxygen had disappeared.

	cub. centims.
Original volume of oxygen dissolved	6·04
Oxygen remaining after 3 hours	5·60
„ „ „ 21 hours	·20
Oxygen absorbed, 3 hours	·44
„ „ 21 hours	5·84

The residual oxygen was estimated by the authors' method (*loc. cit.*, p. 562) after an incubation of usually fourteen hours, sometimes longer.

This period of incubation was sufficient, in the case of those organisms which absorb oxygen rapidly, to ensure the disappearance of all but a trace of the oxygen originally dissolved in the liquid. The numerical results will be found in Table II. in the Appendix.

From the results it is seen that all the aërobic organisms which have been shown to absorb oxygen rapidly by the first method also completely absorb the dissolved oxygen in fourteen hours, whereas others which do not absorb oxygen so rapidly by the first method show a corresponding difference here. A blank experiment made with sterile broth showed a slight absorption owing no doubt to slight air contamination of the water during the aëration process, but this effect is so slight as not to influence the conclusions.

The Necessity of the Presence of Oxygen for the Liquefaction of Gelatine by Anaërobic Organisms.

It was noticed that the only anaërobic organism obtained by a series of broth cultures of crude sewage in hydrogen was one which rapidly liquefied gelatine at the surface only, and it was therefore thought desirable to ascertain whether this surface liquefaction was dependent upon the presence of free oxygen, or whether the organism was capable of liquefying gelatine independently of the presence of oxygen, as it had been found to have the power of growing and multiplying under such conditions.

Accordingly a gelatine culture was made in one of the flasks shown in fig. 1, and hydrogen was passed for half-an-hour through the melted gelatine sown with the organism. The flask was then hermetically sealed and incubated at 22° C.

In twenty-four hours the previously clear gelatine had become uniformly turbid, but *no liquefaction*, such as takes place in twenty-four hours in a gelatine culture exposed to air, had taken place, and even five days' incubation failed to produce the least liquefaction. After this period, air was admitted, and twenty-four hours afterwards a normal liquefaction over the entire surface was in full progress.

Sulphuretted hydrogen was distinctly perceived by smell and proved by its action on lead paper on opening the flask, whilst none is noticed in cultures in air.

Another liquefying anaërobic organism, No. 2, was examined in the same way with a precisely similar result, *i.e.*, no liquefaction of the gelatine took place in hydrogen, even after five days' incubation, but liquefaction set in directly on the admission of air and was in full progress over the entire surface of the gelatine (showing that the liquefying action was not produced by subsequent local air contamination) in twenty-four hours after admission of air. In this case, after twenty-four hours' incubation in hydrogen, the gelatine was riddled with small bubbles of gas, but no putrescent smell or sulphuretted hydrogen was perceived on opening the flask, as was perceived with the last organism.

In the case of Aërobic No. 2, the liquefying fluorescing bacillus, a several days' sojourn in pure hydrogen was found not to be fatal, as liquefaction took place over the entire surface twenty-four hours after opening, although the gelatine had remained quite clear in hydrogen. Evidently the organisms had simply remained dormant during the continuance of these eminently adverse conditions, but still *alive* and waiting for more favourable conditions of environment in order to spring into active growth and multiplication. From this it is evident that some at least of the truly aërobic organisms are able to withstand complete deprivation of oxygen without at once succumbing to the adverse conditions.

Again it is shown in Table I. (Appendix) that a larger amount of oxygen is used up, *cæteris paribus*, when gelatine is liquefied than when the growth takes place in broth alone (see Anaërobic No. 1).

Diminution in the Liquefying Power after long-continued Cultivation in Nutrient Gelatine.

Whether the characteristic modes of growth on nutrient gelatine would remain constant for each subsequent sub-culture of the organism was a subject of experiment. It was found that some at least of the liquefying bacilli lose to a certain extent their power of rapidly liquefying gelatine. In Anaërobic No. 2 this is more especially marked, and Plate 41, figs. 10, *a* and *b*, show this very clearly. Of these *b* is a recently isolated culture, whilst *a* represents a culture of the same age and incubated in the same gelatine side by side with *b*, but from a culture which has repeatedly passed through the process of sub-culture. Hand-drawn records of the early cultures of *a* are found to be identical with fig. *b*. The liquefied gelatine is uniformly turbid and gives a white deposit at the bottom of the liquefied portion, whilst in *a* the great part of the liquefied gelatine remains clear, the growth collecting into little flocculent masses dotted here and there, whilst the liquefied portion is of much smaller extent for the same age. Both cultures are three days old. Aërobic No. 2 also shows this diminishing power of liquefaction. Plate 41, figs. 11, *a*, *b*, *c*, *d*, show the rate of liquefaction in recently isolated cultures, whilst *e* and *f* show the greatly diminished rate of liquefaction of an old culture. For ages see description of plates.

From these observations it is evident that slight differences in the rate of liquefaction or rapidity of growth which may be observed in cultures which give otherwise identical microscopic and macroscopic appearances, should *not* be relied upon for assuming such differing cultures to be distinct species and naming them by different names accordingly.

It would seem that the previous history and environment of the individual organisms which furnish pure cultures is an important factor in determining the precise nature of the manifestations of its growth in gelatine.

It should be remembered also that non-liquefying organisms are liable to undergo changes of a similar character during extended artificial cultures on gelatine. The two widely differing varieties of non-liquefying fluorescing organisms described in this paper are evidence of such change taking place, as both forms were obtained from a single pure culture tube.

Resistance of Spores to Heat.

A few drops of a broth culture of Aërobic No. 4, containing large numbers of spores, were sown into five broth tubes, which were afterwards plunged into water at 80°, 85°, 90°, 95°, and 100° C. for 10 minutes. On incubating the tubes, it was found that the first two tubes alone developed and became crowded with the organism, whilst those heated to 90°, 95°, and 100° C. remained clear and limpid. This is of interest, as the spores of this organism are supposed to be able to withstand a temperature of 100° C.

Further experiments were made by introducing 5 cub. centims. of crude sewage

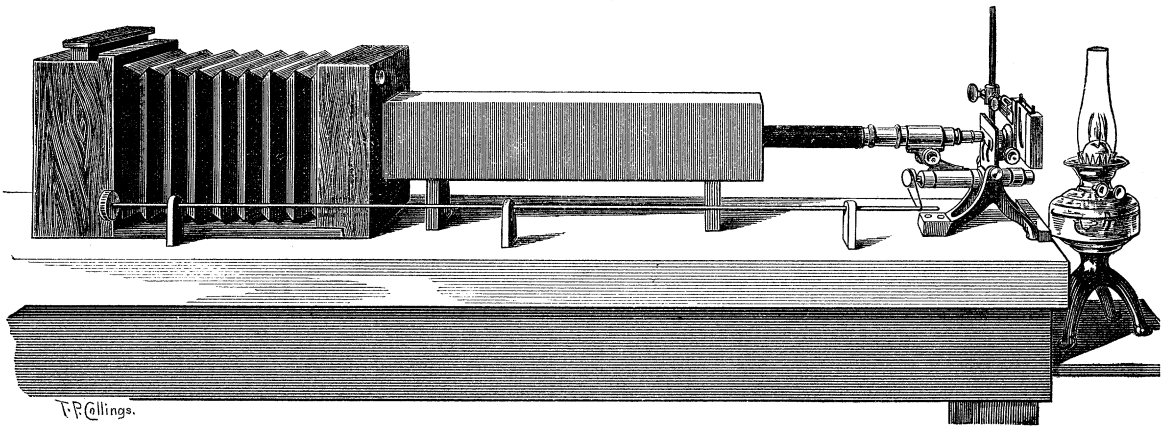
into each of six sterile broth tubes, and placing three in water at 80° C. for 10 minutes, whilst the other three were immersed in water in a state of ebullition for the same length of time. The first three became turbid in two days, and yielded three distinct species of organisms; whilst the other three remained perfectly clear for a fortnight, after which time one of the tubes developed a thin film, and afterwards became slightly turbid. The organism causing this was found to be in *Leptothrix* threads of extreme length; these afterwards split up into shorter spore-bearing rods. This organism has not yet been further studied.

Method employed for Photographing Bacteria.

The authors have adopted the following method in obtaining the photo-micrographs which accompany this paper. It is very simple, and requires no special apparatus or microscopic accessories.

The arrangement of the apparatus is shown in fig. 4.

Fig. 4.



Arrangement for the Photography of Bacteria.

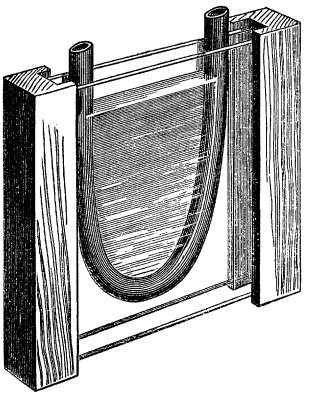
The Illumination.

Source of Light.—A common duplex paraffin oil lamp.

Coloured Screen.—As the stain employed (methyl violet) transmits actinic rays of light, a coloured screen must be employed, in order to obtain actinic contrast on the sensitive plate. The screen selected was spectroscopically adjusted to the stain employed, the object sought being to illuminate the slide with rays which are totally absorbed by the stain used, and which, where unabsorbed by stain, are sufficiently actinic to give a dense negative with short exposure. A weak solution of potassium bichromate was found to serve the purpose admirably. The absorbent liquid is contained in a thin glass trough (fig. 5) interposed between the lamp and the condenser, made by clamping a semicircle of india-rubber tubing between two plates of glass, 4″ × 4″, by means of two pieces of wood grooved to receive the plates. This

solution absorbs all the blue, indigo, and violet of the spectrum, whilst the stain employed absorbs all the yellow and green. The absorption spectra (fig. 6) of these solutions overlap, so that the stained bacteria appear black on a bright yellow background.

Fig. 5.



Bichromate Trough for Yellow Light.

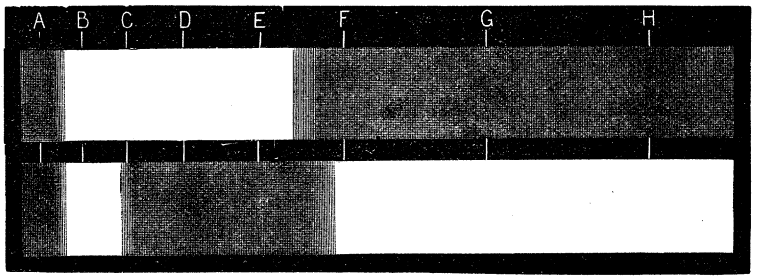
The photographic plates employed must therefore be sensitive to yellow light. Without the interposition of the bichromate screen sufficient actinic light (violet) passes through the stained bacteria to produce a very perceptible action on the photographic plate, thus "fogging" the otherwise sharp and clear outlines of the organisms.

Condenser.—ABBÉ'S condenser is used, without diaphragm, and is focussed rather farther from the object than for ocular examination.

Stage.—A simple clip stage was employed without mechanical accessories.

Fig. 6.

Absorption spectrum of Potassium Bichromate.



Absorption spectrum of Methyl Violet.

The microscopic preparations were obtained, as a rule, from young pure cultures, thus securing cells full of protoplasm, which stain deeply, an essential for actinic contrast on the photographic plate. Wherever possible cover-glass impressions were taken.*

* The authors have extended this mode of preparation to liquids containing mixed or pure cultures with interesting results. Crude sewage, allowed to stand in a covered sterile dish, develops after a while a thin film consisting of various kinds of organisms often aggregated in characteristic pure

Stain used.—Methyl violet was uniformly employed, and has been found to give very satisfactory results in all cases. In spore-bearing rods the spores are left unstained whilst the rods are stained violet.

Mounting medium.—Canada balsam in xylene was uniformly employed. The objections previously entertained to this mounting medium for preparations intended for photography have no weight when the present method of illumination and staining is adopted.

Lenses.—For the photographs magnified 740 diameters a LEITZ' $\frac{1}{12}$th oil immersion was employed. This lens is not usually supposed to be equal to photographic work, yet it may be made to yield very good results. For the photographs magnified 370 diameters a ZEISS' D, and for 50 and 100 a ZEISS' A were used, with the plates at suitable distances from the lens.

No eyepiece was used, nor any lens in the camera, which is connected to the tube of the microscope by a horizontal dark box extension.

Enlargement from the original negative has been resorted to only in the case of the photograph (Plate 43, fig. 8) enlarged to 1500 diameters, in order the more clearly to show spore-formation.

Photographic plates.—EDWARDS' isochromatic plates have been used throughout.

Exposure.—For the image thrown by the immersion lens the exposure is about $1\frac{1}{2}$ minutes, with correspondingly shorter exposures for ZEISS' A and D. The photographs were taken under the disadvantage of vibrations from the vehicular traffic in the street below interfering with the work.

BACTERIOLOGICAL DESCRIPTION OF THE ANAËROBIC ORGANISMS ISOLATED, *i.e.*, THOSE WHICH ARE CAPABLE OF GROWING IN COMPLETE ABSENCE OF FREE OXYGEN.

Anaërobic Organism No. 1.
Proteus vulgaris.

This organism was isolated from sewage by a series of broth cultures in hydrogen. The third hydrogen flask, on examination by plate culture, gave this organism only.

Plate Cultivation.—Plate 42, fig. 3, × 100 diams.

In about twenty hours the whole surface of the gelatine plate is covered by a network of swarmers which branch out in all directions. The filmy net-like coating on the surface is too thin and transparent to yield a photograph by ordinary means, the curious structure of the film being almost invisible to the naked eye, so recourse was had to the following method by which the organisms are *stained on the gelatine*

colonies. To prepare the impressions a clean sterile cover glass is gently placed on the surface of the liquid by sterile forceps, and withdrawn by tilting up one edge to allow the water to recede without disturbance. The film is dried and stained in the usual way. Plate 43, fig. 3, shows an example of a preparation made in this manner. A pure colony was thus obtained direct from crude sewage!

plate itself. The plate is first flooded with absolute alcohol; this kills the organisms and fixes them on the dehydrated and hardened gelatine; then an aqueous alcoholic solution of methyl violet is poured on for a few minutes, until the organisms are stained, when the staining fluid is poured off. The plate is now washed once or twice with water and allowed to dry spontaneously. Differential staining takes place, the organisms stain deeply, whilst the gelatine takes but little. The plate which yielded the photograph is permanent, and shows the same field still, exactly as on the day the cultivation was made.

In the depths the threads permeate the gelatine like gnarled rootlets and appear more opaque than the surface swarmers. In twenty-four hours, or less if the plate is at all crowded, complete liquefaction ensues. The cultures have an unpleasant sickly odour.

Stab Cultivations.—Plate 41, fig. 6; (*a*) twenty-four hours; (*b*) forty-eight hours; (*c*) five days old.

The gelatine becomes liquefied over the entire surface in twenty-four hours, whilst little or no liquefaction has taken place in the needle track. Sometimes the needle track is bristling with rootlet-like projections into the solid gelatine, and shows a very slight incipient liquefaction at the core, and occasionally liquefaction commences at one or two points below the liquefied surface, these appearing as bulbous projections from the central core, filled with turbid liquid having a thick deposit at the bottom. The liquefaction proceeds rather rapidly in a horizontal direction until the whole of the gelatine becomes liquefied, an opaque white deposit remains at the bottom of the culture tube.

Broth Cultures.—A very slight and faint film at the surface.

Microscopic Characters.—Plate 42, figs. 1 and 2, × 740 diams.

These are best studied by cover-glass impressions from plate cultures about twenty hours old. The periphery of the colonies (fig. 1) where liquefaction has not commenced, shows long streamers pushing forward in curious convolutions, these, later on, split up into short rods (fig. 2), which are almost exclusively present in the central and older part of the colony.

Motility.—The rods are rapidly motile.

*Micrometer Readings.**—Rods about 0·6 μ wide, varying from 0·4 to 0·8 μ, and of various lengths; the short rods are 1·4 μ to 4·8 μ long, and all lengths above this may be met with in the long swarmers, which often reach 56 μ in length.

Growth in Hydrogen in Gelatine.—As before described, this organism, when sown in gelatine in an atmosphere of pure hydrogen, renders the medium turbid in twenty-four hours, but *no liquefaction whatever takes* place even after five days. On opening the flask, sulphuretted hydrogen was detected, both by smell and by its reaction with lead paper. Twenty-four hours after opening the flask, the normal surface liquefaction was in full progress, but the smell of sulphuretted hydrogen disappeared

* The measurements were made with a LEITZ' $\frac{1}{20}$ oil immersion, kindly lent by Dr. KLEIN, and a micrometer, for which one division corresponded to 0·4 μ.

Absorption of Atmospheric Oxygen.—Although this organism grows well in entire absence of oxygen, yet the fact that rapid liquefaction of the gelatine surface exposed to air takes place, seems to indicate that oxygen plays an important part in such liquefaction, and this is found to be so on making the experiment in air in a sealed flask.

A larger absorption of oxygen was found to take place in the case where gelatine was used than where broth alone was used. After seven days' incubation the atmosphere was composed as follows:—

	Gelatine.	Broth.
N	80·00	80·00
O	·21	8·21
CO_2	16·43	8·21
Oxygen absorbed . . .	19·79	11·79

PHOTOGRAPHIC Illustrations.

Plate cultivation, 20 hours old, surface growth stained *in situ* × 100 diams.	Plate 42, fig. 3
Stab cultures (1), 24 hours old, actual size	Plate 41, fig. 6*a*
„ (2), 48 „ „	Plate 41, fig. 6*b*
„ (3), 5 days old, „	Plate 41, fig. 6*c*
Photo-micrograph (1) Edge of colony, showing long rods × 740 diams.	Plate 42, fig. 1
„ „ (2) Showing short rods and two swarmers × 740 diams.	Plate 42, fig. 2

Anaërobic Organism No. 2.

This organism was isolated from sewage by plate culture and is usually present in large numbers.

Plate Cultivation. Plate 40, fig. 1.—The colonies appear to the naked eye in twenty-four hours as microscopic centres of liquefaction fringed by short radiating hair-like projections into the non-liquefied gelatine. In two days the colonies have attained a diameter of 6–8 millims. and appear as liquefied circles with a grey turbidity. No fluorescence is developed.

Stab Cultivations. Plate 41, figs. 7, *a*, *b*, and *c*, 10, *a* and *b*.—These are very characteristic. In twenty-four hours liquefaction has commenced throughout the needle track, and shows a slightly wider liquefied portion at the surface. In two days the liquefied portion becomes somewhat conical whilst an opaque white deposit collects at the bottom of the needle track. Fig. 7, *a*, shows a tube three days old, whilst *b* and *c* show the same tube at five and seven days old respectively. In about nine to ten

days the whole of the gelatine become liquefied, leaving an opaque white deposit at the bottom of the tube. No film is formed at the surface. Fig. 10 shows the diminished power of liquefaction possessed by this organism after repeated sub-cultivation in gelatine. The two tubes were inoculated from completely liquefied tubes of the same age, one a recently isolated culture, the other an old culture. Both new cultures were in tubes of the same gelatine and were incubated for three days side by side. Fig. *a* shows the tube from the old culture; liquefaction has not extended very far; the growth is of a flocculent character and a deposit has fallen to the bottom. Fig. *b*, from the recent culture, shows a thickly turbid liquid; liquefaction has proceeded much more rapidly than in fig. *a*.

Broth Cultures.—No film is formed at the surface.

Agar Agar.—Creamy-white moist growth which does not extend far from the central line.

Potato.—Grows well on potato, giving a brownish-white moist growth.

Microscopic Characters. Plate 42, figs. 4 and 5, × 740 diams.—The early cultures of this organism showed mainly short rods with occasional very long rods (fig. 4). In old cultures, after repeated sub-cultures in gelatine, not only does the organism show a diminished power of liquefying gelatine but the microscopic characters also become greatly changed. Involution forms of all kinds appear, distinctly celled chains of rods of varying length and thickness, extremely long tapering rods often with club-shaped ends, rods of very various lengths singly and in short chains of three or more (fig. 5).

Motility.—The rods are rapidly motile.

Micrometer Readings.—The rods are at first from 0·4 μ to 0·6 μ wide, and of variable length from bacillo-cocci to very long rods and chains.

Growth in Hydrogen in Gelatine.—Sown in gelatine, in an atmosphere of pure hydrogen, the gelatine becomes turbid after twenty-four hours' incubation, and also riddled with minute bubbles of gas, but *no liquefaction whatever* takes place even after five days' incubation in hydrogen; twenty-four hours after opening the flask, however, the whole surface becomes liquefied under the influence of the oxygen which is admitted. In broth, in hydrogen, the organism grows as very short rods, scarcely longer than broad. No objectionable odour or sulphuretted hydrogen is perceived, either before or after access of air.

Absorption of Atmospheric Oxygen.—A slight absorption only takes place. After seven days' incubation of 25 cub. centims. of infected broth in 250 cub. centims. of air, the atmosphere possessed the following composition :—

N	80·00
O	17·60
CO_2	3·80
Oxygen absorbed	2·40

PHOTOGRAPHIC Illustrations.

Plate cultivation	2 days old, actual size	Plate 40, fig. 1
Stab cultures (rather old sub-culture) (1),	3 ,, ,,	Plate 41, fig. 7*a*
,, ,, ,, (2),	5 ,, ,,	Plate 41, fig. 7*b*
,, ,, ,, (3),	7 ,, ,,	Plate 41, fig. 7*c*
,, ,, ,, (4), Old culture	3 days	Plate 41, fig. 10*a*
,, ,, ,, (5), New culture	3 days	Plate 41, fig. 10*b*
Photo-micrograph, new culture, short rods × 740 diams. . .		Plate 42, fig. 4
,, ,, old ,, involution forms × 740 diams.		Plate 42, fig. 5

Anaërobic Organism No. 3.

Streptococcus mirabilis.

This organism was isolated from sewage by the dilution method, and appeared in a broth flask, accompanied by a bacillus, from which it was separated by plate culture.

Plate Cultivation. Plate 40, fig. 2, × 50 diams.—This organism grows badly in gelatine, and even after four days the colonies appear in the depths of the gelatine as mere microscopic dots or gnarled and convoluted thread-like masses. Some surface colonies, especially in the early cultures, show an exceedingly faint and transparent expansion about 2 millims. in diameter; this examined microscopically with a low power is seen to consist of a mass of fine long threads, sometimes throwing out processes into the surrounding gelatine. These colonies are not amenable to cover-glass impressions, as they obstinately refuse to leave the gelatine. Fig. 2 shows a photograph (× 50 diams.) of a preparation of a colony obtained as follows:—The gelatine plate was flooded with dilute nitric acid, which dissolved the gelatine and set the colony free, this was then gently floated on a cover-glass, where it was left stranded, the colony was then washed and dried and stained in the usual manner. Plate 42, fig. 8, shows a small portion of the edge of this colony × 370 diams., and well illustrates the loops which spring out into the adjacent gelatine. No liquefaction of the gelatine takes place even in old cultivations, and growth apparently ceases after the first five or six days.

Streak Cultures on Gelatine. Plate 41, fig. 1.—An exceedingly faint and transparent film, almost invisible to the naked eye, which grows for a few days, attaining a diameter of about 3–5 millims., at which point further growth ceases. A very characteristic culture is obtained in the following manner:—a streak culture on gelatine is made, and when seven days old the tube is filled up with sterile broth, submerging the gelatine. The streak on the gelatine soon begins to throw out fluffy

loops of threads into the broth. The figure (fig. 1) shows such a culture, grown seven days in gelatine, and a further seven days in the broth. The growth stands out from the gelatine into the broth as a fine fluffy mass, resembling delicate cotton wool; the gelatine is shown on the right, whilst the broth on the left, above the fluffy growth, remains perfectly clear and limpid.

Broth Cultures.—These are very characteristic. In forty-eight hours nearly the maximum growth has taken place; a fine mass resembling delicate cotton-wool rests at the bottom of the test-tube, or is carried upwards in delicate festooned threads by the convection currents in the liquid. When the tube is taken out of the incubator, and convection currents cease, the festooned threads fall to the bottom, leaving the broth above perfectly clear and limpid. It is a characteristic of this organism, owing to its growing in such interminably long threads, that the broth in which it grows is not rendered turbid in the least.

Agar Agar.—The growth on this medium is very similar to that on gelatine, being a very faint slight expansion.

Potato.—Inappreciable growth.

Microscopic Characters. Plate 42, figs. 7 and 8.—Streptococci in chains of extreme length, rarely splitting off into short threads, hence the non-turbidity of the broth cultures.

Motility.—The organism is non-motile.

Micrometer Readings.—The chains are about 0·4 μ thick, and the individual cells vary from 0·4 μ in the single cells to about 1·2 μ in the diplococci undergoing fission.

Growth in Broth in Hydrogen.—The organism grows quite as readily in pure hydrogen as in air, and with the same characteristic appearance.

Absorption of Atmospheric Oxygen.—After seven days the absorption of oxygen is almost *nil*, and is the smallest of any result obtained. The atmosphere gave on analysis the following numbers:—

N	80·00
O	19·50
CO_2	·30
Oxygen absorbed	·50

Photographic Illustrations.

Colony 7 days old, preparation by nitric acid method described, × 50 diams.	Plate 40, fig. 2.
Streak culture, 7 days on gelatine and 7 days in broth . . .	Plate 41, fig. 1.
Photo-micrograph, small portion of edge of above colony, × 370	Plate 42, fig. 8.
,, ,, preparation of broth culture 24 hours old, × 740	Plate 42, fig. 7.

Anaërobic Organism No. 4.
Bacillus opalescens.

This organism was isolated from sewage direct by plate culture; it occurs plentifully in the sewage experimented with.

Plate Cultivation. Plate 40, fig. 3.—The colonies are easily visible as small faint expansions after twenty-four hours' incubation, diameter 1–2 millims. In two days they are very characteristic, appearing as a thin opalescent film, about 8 millims. diameter, with an irregular wavy edge of extreme thinness. The colonies are admirably adapted for cover-glass impressions, the edge showing a single layer of well-defined rods. No liquefaction takes place even in old cultures. No fluorescence is produced. The colonies seem to have an aversion to coalesce with each other; the edge of a colony near its neighbour grows less quickly, whilst the edges which have a free course shoot out more quickly into the unoccupied space.

Streak Cultures.—Plate 41, fig. 2, shows a streak two days old. The gauzy and irregular margins are well seen; the film is of an opalescent character.

Stab Cultures.—The growth is confined to the surface, which is covered completely in three or four days, when the growth ceases. No liquefaction whatever takes place.

Agar Agar.—A creamy white moist growth which does not extend far from the central line.

Potato.—Grows well on potato, giving a brownish-white slimy growth.

Microscopic Characters. Plate 43, figs. 1 and 2.—These are best studied by cover-glass impressions. The delicate edges of the colonies are seen to be formed by a single layer of well-defined rods of uniform length; these divide in the older stages into short rods, and even cocci. The photographs show all the forms from one cover-glass impression. Fig. 1 shows the long rods from the edge of a colony, whilst fig. 2 shows the uppermost layer of organisms from the centre of the colony.

Motility.—This organism is motile.

Micrometer Readings.—The long rods measure about 0·5 μ wide and 4 μ long, whilst the older forms may be cocci 0·4 μ in diameter.

Growth in Hydrogen.—The broth becomes turbid in twenty-four hours, but the growth is not so copious as in air. After a few days a white sediment falls to the bottom. Here the cocci forms predominate.

Absorption of Atmospheric Oxygen.—The absorption of oxygen by this organism is not very rapid. After seven days the atmosphere possessed the following composition:—

N	80·0
O	13·3
CO_2	6·3
Oxygen absorbed	6·7

PHOTOGRAPHIC Illustrations.

Colonies on gelatine, two days old	Plate 40, fig. 3.
Streak culture on gelatine, two days old	Plate 41, fig. 2.
Photo-micrograph (1) Long rods, edge of colony, × 740 diams.	Plate 43, fig. 1.
,, ,, (2) Short rods and cocci, centre of colony, × 740 diams.	Plate 43, fig. 2.

Anaërobic Organism No. 5.

This organism was isolated from sewage-infected broth incubated in hydrogen, by the method for isolating spore-forming organisms.

Plate Cultivation.—The colonies appear on the second day as tiny translucent droplets, they never exceed 2 to 3 millims. in diameter, when further growth ceases. Owing to its very difficult growth on gelatine this organism has not yet been completely examined.

Streak Cultivation.—A faint translucent and very narrow streak, about 1–2 millims. wide, develops after two days, but no further growth takes place. No liquefaction of the gelatine takes place.

Broth Cultures.—These also grow badly.

Microscopic Characters.—Plate 42, fig. 6, shows a cover-glass impression from an isolated colony. This has been so treated in the heating and washing, that only the first layer of organisms has been fixed on the cover-glass, the lower layers, which would otherwise make the preparation quite opaque, have all been washed away from the preparation without disturbing the first layer. The wave-like arrangement of the rods is very marked.

Micrometer Readings.—The rods are 0·5 to 0·8 μ wide and 1·8 to 5·2 μ long.

Plate 40, fig. 5, shows this cover-glass impression viewed in its entirety by a low power, with dark ground illumination. The mottled appearance is very curious. It is caused by the arrangement in different directions of the single layer of rods, whereby, in one direction, light is able to pass, whereas, in the other it is not.

PHOTOGRAPHIC Illustrations.

Photo-micrographs, cover-glass impression, three days old, dark ground illumination, showing mottled appearance, × 50 . .	Plate 40, fig. 5.
Ditto, more highly magnified, × 370	Plate 42, fig. 5.

Bacteriological Description of Aërobic Organisms isolated, *i.e.*, those which are incapable of growing except in presence of free Oxygen.

Aërobic Organism No. 1.
Bacillus fluorescens non-liquefaciens (A).

This organism was isolated from sewage by plate culture.

Plate Cultivation. Plate 40, fig. 7*a*.—The colonies are easily visible to the naked eye as small transparent expansions on the surface of the gelatine after twenty-four hours' incubation. In two days the surface colonies have attained a diameter of 3–3·5 millims., the edges are very delicately thin and irregular, whilst the central and older portion is thicker and of a slightly grey colour. These colonies are admirably adapted for the preparation of cover-glass impressions, the margins showing a delicate single layer of well-defined rods.

On the second day the colonies are surrounded by a broad halo of beautiful bluish-green fluorescence. After three or four days' incubation, softening and incipient liquefaction of the gelatine commences; this proceeds very slowly and not at all if grown at the ordinary temperature. In old cultures the bluish-green fluorescence fades, and is replaced by a brown colour.

Streak Cultivations, Gelatine. Plate 41, fig. 4*a*.—The streak grows rapidly for two or three days, after which time the growth proceeds but slowly. Fluorescence is very marked after two days' growth. The photograph well shows the gauzy irregular margin of the growth and the incipient softening of the gelatine in the central portion.

Broth Cultures.—A slight film is formed at the surface and the broth is tinged a fluorescent green.

Agar Agar.—A greyish-white growth which does not extend far from the central line. The whole of the agar agar is tinged a fluorescent green.

Potato.—A creamy white growth which soon turns brown, and does not extend over the entire surface.

Microscopic Characters. Plate 43, fig. 6.—Thin motile rods, longer and slightly thicker in the early stages (edge of colony), than in the later ones. This organism does not form chains, and very rarely long rods, and is identical in cover-glass impressions with Plate 43, fig. 6.

Motility.—The rods are motile.

Growth in Hydrogen.—This organism refuses to grow in an atmosphere deprived of oxygen.

Absorption of Atmospheric Oxygen.—This organism is a greedy absorber of gaseous oxygen. In one experiment a 25 cubic centims. broth culture sealed up in 250 cubic centims. of air, deprived the atmosphere of all but a trace of oxygen during seven days' incubation. The analysis of the gases gave the following:—

N	80·00
O	·14
CO_2	12·04

In another experiment made side by side with the next-described organism the results were, for seven days :—

N	80·0
O	7·9
CO_2	8·6

The diminished rate of absorption is not easily explainable except by the fact that the second experiment was sown from an older and perhaps weaker culture.

Photographic Illustrations.

Plate cultivation, 2 days old, actual size	Plate 40, fig. 7*a*.
Streak cultivation, 3 days old	Plate 41, fig. 4*a*.
Photo-micrograph, cover-glass impression, edge of colony, × 740 (identical with)	Plate 43, fig. 6.

Aërobic Organism No. 1A.
Bacillus fluorescens non-liquefaciens (B).

This organism, evidently a variety of the one last described, was isolated from a culture of that organism which had repeatedly passed through plate culture, and which, therefore, was undoubtedly pure. On making a fresh plate cultivation from a streak tube it yielded two varieties of fluorescent colonies, each of which retained its character in subsequent cultures. The organisms are strikingly alike in their fluorescent power, but distinct in their gelatine cultures and in microscopic preparations. The authors believe that here some subtle change has taken place in a portion of a pure culture by which the character of the organism is altered, thus giving rise to two varieties. This variety has also been isolated direct from sewage.

Plate Cultivation. Plate 40, fig. 7*b*.—In two days, plates side by side with those of the first variety *grew less quickly*, the colonies not exceeding 2 millims. in diameter; the outline of these is quite sharp and *circular*, the edges being much thicker in contrast to the first variety with its gauzy margins and irregular outline. The colonies are thicker and whiter in colour, and show the same fluorescence as the first variety.

Streak Cultivation. Plate 41, fig. 4*b*.—These show a marked contrast to 4*a*, as is seen very well in the photograph. Incipient liquefaction does not commence until the fifth day's incubation, whilst in 4*a* it is present on the third day.

Stab Cultivations.—The surface growth is like the colonies, a circular head; there

is little growth in the needle track; the fluorescence extends throughout the whole of the gelatine after about a week's incubation.

Agar Agar and Potatoes.—Similar to the preceding organism.

Microscopic Characters. Plate 43, fig. 3.—Here the difference is marked; this variety is encapsuled, whilst the first variety is not.

Growth in Hydrogen.—This organism refuses to grow in an atmosphere deprived of oxygen.

Absorption of Atmospheric Oxygen.—The experiment made side by side with the one last named gave the following numbers:—

N	80·0
O	4·9
CO_2.	10·1

PHOTOGRAPHIC Illustrations.

Plate cultivation, 2 days old, actual size	Plate 40, fig. 7*b*.
Streak cultivation, 3 days old, actual size.	Plate 41, fig. 4*b*.
Photo-micrograph, encapsuled rods direct from sewage (see p. 643, footnote) × 740 diams	Plate 43, fig. 3.

It was considered desirable to expose crude sewage to a large surface of air and allow the natural changes to go on for some time, in order to ascertain the nature of the organisms which would thrive under those conditions. For this purpose crude sewage was introduced into a sterilised and covered Petri's dish and allowed to remain for two months. After this length of time cover-glass impressions and plate cultures were made from the liquid, when it was found that the former showed plentiful pure colonies of encapsuled rods.

Plate 43, fig. 3, shows a photograph of such a colony; the plate cultivations too yielded the non-liquefying fluorescent colonies and encapsuled rods described above.

This organism and the preceding closely correspond to the organisms isolated by Dr. KLEIN from poisonous veal and pork.

Aërobic Organism No. 2.
Bacillus fluorescens liquefaciens.

This organism was isolated from sewage by plate culture direct, it occurs plentifully in sewage.

Plate Cultivation. Plate 40, figs. 10 *a* and *b*.—The colonies develop with extreme rapidity. In twenty-four hours they appear as slightly fluorescent liquefied circles 2-3 millims. in diameter, and grow so rapidly as to cover 4–5 millims. in another three

hours. It is interesting to observe that the colonies submerged in the gelatine appear as mere dots during this extremely rapid liquefaction taking place in the surface colonies, showing the accelerative effects of a copious supply of oxygen. A successful plate must contain very few colonies or complete liquefaction of the whole of the gelatine soon takes place.

Stab Cultivations. Plate 41, figs. 11, *a* to *d*.—The liquefaction stretches half across the surface of the gelatine in twenty-four hours, forming a curved turbid portion beneath (fig. *a*). In twenty-seven and a half hours the liquefaction has extended almost across the surface, whilst the needle track in the depths remains undeveloped (fig. *b*). Figs. *c* and *d* show the further course of the liquefaction. After two or three days the liquid shows a greenish fluorescence which becomes more marked in old cultures.

Microscopic Characters.—Short motile rods corresponding to Plate 42, fig. 4, length variable, width 0·4 μ to 0·7 μ.

Absorption of Atmospheric Oxygen.—This organism absorbs oxygen rapidly; in one experiment the growth abstracted nearly all the oxygen in three days, and in another of seven days' incubation the merest trace of oxygen was found. In a later series of experiments, however, the absorption was not so rapid. The following are the numbers obtained :—

	(1) 3 days.	(2) 7 days.	(3) 3 days.	(4) 7 days.
N	80·0	80·0	80·0	80·0
O	1·0	0·0	12·8	4·6
CO_2	10·7	12·7·	5·4	11·4

Growth in Hydrogen.—No growth takes place in gelatine, nevertheless, the organisms retain their vitality for some days at least, and normal liquefaction proceeds on admitting air to the flask.

Photographic Illustrations.

Plate cultivations, 24 hours old, actual size.	Plate 40, fig. 10*a*
,, 27 ,, ,, ...	Plate 40, fig. 10*b*
Stab cultivations, 24 ,, ,, ...	Plate 41, fig. 11*a*
,, 27½ ,, ,, ...	fig. 11*b*
,, 48 ,, ,, ...	fig. 11*c*
,, 4 days old ,, ...	fig. 11*d*
,, old cultures, 3 days old	fig. 11*e*
,, ,, 7 ,, ...	fig. 11*f*
Photo-micrograph × 740 diams. (identical with)	Plate 42, fig. 4

Aërobic Organism No. 3.

Bacillus subtilis.

This organism was isolated from sewage by the method described for isolating spore-forming organisms. A broth tube containing 2 cubic centims. of crude sewage was placed in water at 80° C. for ten minutes, and incubated for two days. After this period of incubation the broth swarmed with this organism, which was obtained in the pure state from this material by plate cultivation.

Plate Cultivation. Plate 40, fig. 9.—The colonies begin to appear as liquefying dots in twenty-four hours; on the second day the surface colonies have attained a diameter of 14–16 millims. They appear as liquefied circles, covered in the central portion by a more or less perfect film. Microscopically, the edges of the colonies exhibit short streamer-like projections into the non-liquefied gelatine, and the colony appears crowded with dark dots, which vanish and reappear, probably due to the rods presenting an end and side view alternately whilst moving about in the liquid. No objectionable odour is perceived.

Stab Cultivations. Plate 41, fig. 8, *a* and *b*.—The liquefaction of the gelatine begins as a hemispherical turbid portion at the point of entrance of the needle. Fig. 8*a* shows a tube twenty-four hours old. On the second day, the liquefaction extends across the tube and for several millimetres downwards, whilst the needle track below has become liquefied and has widened out. A white firm film is formed on the surface consisting of matted threads of the organism in a non-motile condition. After about seven days this film is crowded with spores.

The whole of the gelatine becomes liquefied after 7–10 days when a somewhat flocculent deposit is formed at the bottom of the tube, whilst the liquid beneath the film becomes comparatively clear.

Broth Cultures.—In these a firm film forms on the surface in three or four days, and this falls to the bottom when shaken.

Microscopic Characters. Plate 43, figs. 7 and 8.—Bacilli singly in twos, threes, and short chains, which sometimes may consist of more than a dozen elements. Fig. 7 is a photograph of a cover-glass impression, from the fourteen days old broth which had been poured out into a sterile dish, from the sealed flask used for the determination of the absorption of oxygen. The preparation was made the day after pouring into the dish, and curiously enough shows mainly long chains. The short chains, however, consisting of but two or three elements, are more characteristic of the gelatine cultures. Spore formation is observed after seven days' incubation, and Plate 43, fig. 8, shows an enlargement × 1500 diameters, taken from a photograph × 740 diameters. The spores were not stained in the preparation, and come out white in the photograph.

Motility.—In the fresh state the rods are motile, whilst the zooglea film is a mass of interlacing non-motile rods, which soon forms spores.

Micrometer Readings.—Rods 0·5 μ to 1 μ wide and 3 μ to 5 μ long. The spores measure about 0·8 μ wide and 1·2 μ long, and are often thicker than the rods bearing them.

Growth in Hydrogen.—No development takes place, but a fourteen days' deprivation of oxygen was found not to be fatal, as on the second day after opening to the air, the liquid swarmed with the rapidly motile rods.

Absorption of Atmospheric Oxygen.—Rapid absorption of oxygen takes place. After seven days and fourteen days respectively, two experiments gave :—

Seven days.		Fourteen days.	
N	80·0	N	80·0
O	9·0	O	0·8
CO_2	8·8	CO_2	14·1
Oxygen absorbed . .	11·0	Oxygen absorbed . .	19·2

Resistance of the Spores to Heat.—From a completely liquefied gelatine tube, crowded with spores, sowings were made in five tubes of sterile broth, and the tubes afterwards placed for ten minutes in water at 80°, 85°, 90°, 95°, and 100° C., respectively. They were then allowed to cool and placed in the incubator. After two days, the 80° and 85° tubes became turbid, and afterwards developed the characteristic film and rods of the organism. Spore-formation also took place. The 90°, 95°, and 100° C tubes all remained perfectly clear and limpid.

Photographic Illustrations.

Plate cultivation, 2 days old, actual size	Plate 40, fig. 9
Stab cultures, 24 hours old „ „	Plate 41, fig. 8*a*
„ 2 days „ „ „	Plate 41, fig. 8*b*
Photo-micrographs, *Leptothrix* threads, × 740 diams. . . .	Plate 43, fig. 7
Enlargement, spore-bearing rods, × 1500 diams.	Plate 43, fig. 8

Aërobic Organism No. 4.

Bacillus violaceus.

This organism was isolated from sewage by plate cultivation.

Plate Cultivations. Plate 40, fig. 8.—The colonies closely resemble those of Anaërobic Organism No. 4. They grow, however, rather more slowly, and the films are thicker and more opaque, and the edges are not so delicate. After five or six days' growth the colonies assume a deep violet colour, and in this manner they are sharply distinguished from the organism mentioned.

Streak Culture. Plate 41, fig. 3.—The photograph shows the appearance of a streak

culture, three days old; in this, the violet colour has not yet appeared, but the growth is yellowish-white. After five or six days the colour begins to turn violet, and soon afterwards liquefaction of the gelatine sets in, and ultimately involves the whole of the gelatine, whilst a rather thick deposit of a dirty violet colour collects at the bottom of the tube.

Broth Cultures.—A thick film of a dirty violet colour is formed, whilst the liquid is coloured brown.

Agar Agar.—A grey-white moist growth, which gradually assumes a bluish-violet colour, which is especially marked at the surface of the liquid collected at the bottom of the tube. The deposit at the bottom of the liquid is white.

Potato.—A thick moist brown expansion with old cultivation.

Microscopic Characters. Plate 43, fig. 6.—Bacilli corresponding microscopically to Aërobic No. 1. Fig. 6 is from a photograph of a cover-glass impression showing the edge of a colony.

Motility.—The organism examined in the living state is motile.

Growth in Hydrogen.—No growth takes place.

Absorption of Oxygen.—Like all the film-forming organisms, this absorbs oxygen rapidly. After seven days the atmosphere was almost deprived of oxygen.

N	80·00
O	·14
CO_2	12·06
Oxygen absorbed	19·86

PHOTOGRAPHIC Illustrations.

Plate cultivation, 2 days old, actual size	Plate 40, fig. 8.
Streak cultivation, 3 days old, actual size	Plate 41, fig. 3.
Photo-micrograph, edge of colony, × 740 diams.	Plate 43, fig. 6.

Aërobic Organism No. 5.
Streptococcus ureæ.

This organism was isolated from sewage direct by plate culture.

Plate Cultivation. Plate 40, fig. 6.—The colonies begin to appear on the second day as minute dots; on the third day the surface colonies have acquired a diameter of 2 millims., and on the fifth day (fig. 6) the increase is only to 3 millims.; from this point the growth proceeds very slowly. No liquefaction takes place even in old cultures. In contour the colonies are well-defined circles, and appear as shining drops of wax of an opaque yellowish-white colour.

Streak Cultivations.—Plate 41, fig. 5, shows a cultivation three days old. After this

point the further growth takes place very slowly, giving a straight waxy streak. No liquefaction ever takes place.

Stab Cultures.—A well-defined circular head, corresponding to the appearance on plates, develops at the point of entrance of the needle. Little growth is noticed in the needle track.

Agar Agar.—A grey-white moist growth, which does not extend far from the central line.

Potato.—A creamy-yellow thick growth.

Microscopic Characters. Plate 43, fig. 4.—This organism is apparently a streptococcus, growing in short chains, and in ones and twos. The preparation is from a young and vigorously growing cultivation, and shows all the transition stages from the true streptococci through chains of oval and then bacilli-like cells, which subsequently split off into cocci.

Motility.—This organism is non-motile.

Micrometer Readings.—The cells are 0·8 μ to 1·2 μ wide, and 0·8 μ to 2·4 μ long, according to stage.

Growth in Hydrogen.—No growth was observed.

Absorption of Atmospheric Oxygen.—The growth in broth is not so rapid and the turbidity not so dense as in the case of other organisms, and the absorption of oxygen takes place but slowly. After seven days the atmosphere was composed as follows :—

N	80·0
O	14·0
CO_2	3·6
Oxygen absorbed	6·0

PHOTOGRAPHIC Illustrations.

Plate cultivation, 5 days old, actual size.	Plate 40, fig. 6.
Streak cultivation, 3 days old, actual size	Plate 41, fig. 5.
Photo-micrograph, showing all the transition stages in streptococci, × 740 diams.	Plate 43, fig. 4.

Aërobic Organism No. 6.
Micrococcus.

This organism was isolated by plate cultivation from sewage direct. It is interesting to note that it is the only micrococcus met with.

Plate Cultivation. Plate 40, fig. 4.—This organism grows very slowly, the colonies appearing as minute dots on the second day. The first crowded plate is viscid and possesses a sickly odour. On the fifth day the colonies have only advanced to 2 millims. diameter. Microscopically, the colonies appear as dark well-defined spheres. Incipient liquefaction commences about the fifth day and proceeds but slowly ; the

gelatine only becomes viscid and not really liquid. The colour of the growth is a pale yellowish-brown.

Stab Cultivations. Plate 41, figs. 9, *a* and *b*.—There is but little growth in three days, when a slight viscid depression is formed at the entrance of the needle; but little growth takes place in the needle track. The photographs show stab cultivations seven and fourteen days old respectively.

Agar Agar.—A slight yellow growth, showing a tendency to collect in droplets.

Potato.—A very poor growth of a dry yellow crumpled nature.

Microscopic Characters.—Cocci 0·5 μ to 0·8 μ diam., which exhibit only the usual vibratory movements.

Absorption of Atmospheric Oxygen.—The absorption is very slow, corresponding to that given by Anaërobic No. 2. After seven days' incubation the results were :—

N	80·0
O	17·6
CO_2	5·8
Oxygen absorbed	2·4

PHOTOGRAPHIC Illustrations.

Plate cultivation, 5 days old, actual size	Plate 40, fig. 4
Stab cultivations, 7 ,, ,, ...	Plate 41, fig. 9*a*
,, 14 ,, ,, ...	Plate 41, fig. 9*b*

Résumé.

Of the twelve organisms studied, five are capable of growing in complete absence of free oxygen, whilst to the other seven free oxygen is a necessity.

Of the five organisms which can grow without free oxygen, one only, the first one, shows a vigorous absorption of this gas, and this is the one which gives rise to offensive decomposition of the nutrient material.

Of the seven organisms to which free oxygen is a necessity, five are rapid absorbers of that gas, and all of them form films of a more or less marked character when cultivated in nutrient broth, and it would seem that the chemical signification of such film-forming at the surface, *is*, that the organisms require and absorb a large amount of free oxygen. These organisms therefore may be regarded as a means of slow combustion and destruction of the organic matter of sewage.

The other two organisms which do not form films absorb oxygen much less rapidly.

Of the twelve organisms, four rapidly liquefy gelatine, and of these two are anaërobic and two aërobic. Four liquefy gelatine slightly after lapse of several days' cultivation. These are all aërobic. The remaining four do not liquefy the gelatine at all, even in old cultivations. Nine of the organisms belong to the class of Bacilli, whilst two are Streptococci and one a Micrococcus.

Appendix.

Table I.—Showing the Absorption of Oxygen from the Atmosphere of Sealed Flasks (fig. 2), containing 25 cub. centims. of Nutrient Broth, and 250 cub. centims. of Air, and sown with Traces of Pure Cultures of the Organisms. Temperature of Incubation, 20°–23° C.

A. Anaërobic Organisms.

Organism	No. 1. (*Broth.*)	No. 1. (*Gelatine.*)	No. 2.	No. 3.*	No. 4.
Duration of incubation	7 days	7 days	7 days	7 days	7 days
Composition of residual atmosphere: N	80·00	80·00	80·0	80·0	80·0
Composition of residual atmosphere: O	8·21	·21	17·6	19·5	13·3
Composition of residual atmosphere: CO_2	8·21	16·43	3·8	·3	6·3
Oxygen absorbed per 80 parts nitrogen	11·89	19·79	2·4	0·5	6·7

B. Aërobic Organisms.

Organism	No. 1.	No. 1.	No. 1a.	No. 2.	No. 2.	No. 2.	No. 2.
Duration of incubation	7 days	7 days	7 days	3 days	7 days	3 days	7 days
Composition of residual atmosphere: N	80·00	80·0	80·0	80·0	80·0	80·0	80·0
Composition of residual atmosphere: O	·14	7·9	4·9	1·0	trace	12·8	4·6
Composition of residual atmosphere: CO_2	12·04	8·6	10·1	10·7	12·7	5·4	11·4
Oxygen absorbed per 80 parts nitrogen	19·86	12·1	15·1	19·0	20·0	7·2	15·4

Organism	No. 3.	No. 3.	No. 4.	No. 5.	No. 6.
Duration of incubation	7 days	14 days	7 days	7 days	7 days
Composition of residual atmosphere: N	80·0	80·0	80·00	80·0	80·0
Composition of residual atmosphere: O	9·0	·8	·14	14·0	17·6
Composition of residual atmosphere: CO_2	8·8	14·1	12·06	3·6	5·8
Oxygen absorbed per 80 parts nitrogen	11·0	19·2	19·86	6·0	2·4

* This result may be taken as a blank experiment to show that no appreciable absorption takes place with pure broth and air when sterile.

TABLE II.—Showing the Absorption of Dissolved Oxygen from Sterilised Tap Water containing a definite amount of Free Oxygen in solution, after sowing with 1 per cent. of a two days old Broth Cultivation, and incubating for a definite time. Temperature of Incubation, 21–23° C.

Anaërobic Organisms.

Organism	No. 3.	No. 4.	Blank expt.
Original volume of oxygen dissolved	6·04	6·04	6·04
Residual oxygen	4·70	·12	5·10
Oxygen absorbed	1·34	5·92	·94
Duration of incubation . . .	14 hours	14 hours	14 hours

Aërobic Organisms.

Organism	No. 1.	No. 1A.	No. 2.	No. 3.	No. 4.
Original volume of oxygen dissolved	6·04	6·04	6·04	6·04	6·04
Residual oxygen	·05	·07	·15	·51	5·60
Oxygen absorbed	5·99	5·97	5·89	5·53	·44
Duration of incubation . . .	14 hours	14 hours	14 hours	14 hours	3 hours

Organism	No. 4.	No. 4.	No. 5.*	No. 6.	No. 6.
Original volume of oxygen dissolved	6·04	6·04	6·04	6·04	6·04
Residual oxygen	·20	·07	·20	4·00	1·32
Oxygen absorbed	5·84	5·97	5·84	2·04	4·72
Duration of incubation . . .	21 hours	14 hours	14 hours	14 hours	42 hours

PREPARATION OF STERILE GELATINE TUBES AND METHOD OF PLATE CULTURE USED.

As the method of preparing sterile gelatine tubes used by the authors is somewhat simpler and shorter than that usually adopted, the details are here appended.

(1) The test tubes, 5 or 6 inches × $\frac{3}{4}$, are first washed and set up on end to drain and then heated to 150° for an hour in the steriliser.

(2) Pure cotton-wool is placed in a *steam* steriliser and subjected to a current of

* Four days old broth (slow-growing organism).

wet steam for two hours, and afterwards dried in the *hot air* steriliser by heating to 150° C. for half an hour. This method of sterilising the wool is to be preferred to the usual one of heating in the hot air steriliser for several hours on several successive days, not only on account of the saving in time, but also because it gives a whiter wool without brittle and partly charred threads. The treatment is quite effective, steam at 100° C. being a better germicide than hot dry air at the same temperature. By this method sterile plugged tubes can be prepared in one day instead of requiring several days.

(3) The sterile tubes are now plugged in the usual way, using clean fingers to manipulate the wool. When about half a gross of tubes are thus plugged they are again placed in the hot air steriliser and raised to 150° C. for an hour.

Preparation of Sterile Nutrient Gelatine.—One pound of lean beef free from fat is finely minced and placed in a large beaker with clock glass cover. A litre of tap water is poured over the mass and the whole placed in the steam steriliser for one and a half hour, stirring the mass to mix thoroughly after the first half hour. After one and a half hour's steaming the liquid is filtered into another large beaker containing—

100 grms. of gelatine.
10 grms. of peptone.
5 grms. of salt.

The hot filtrate quickly dissolves the gelatine, and the mass should be stirred until the sheets are broken up and dissolved. The mixture is now placed in the steam steriliser for half an hour, neutralised with potassium carbonate, and replaced in the steriliser for another hour. The turbid fluid is now filtered into a large flask without any sterilising precautions whatever, and distributed in the usual manner to the sterile plugged tubes. The half gross of plugged and filled tubes are now placed together in a potato steamer and steamed for fifteen minutes. Steaming is repeated on the second and third day for ten minutes each day.

The authors find this treatment quite effective, the tubes obtained never show the least growth on keeping. Out of many hundreds of tubes only two have shown any growth, and both, on examination, proved to be cracked tubes, and probably the cold water bath, in which the tubes are placed to set, introduced organisms through the crack.

Before opening the tubes the tuft of wool was uniformly singed to burn up the dust and germs which might have fallen on the outside.

Preparation of Sterile Peptone Broth.—For this the 100 grms. of gelatine is omitted, otherwise exactly the same method is used.

Preparation of Sterile Agar Agar.—For the 100 grms. of gelatine 20 grms. of agar agar are employed.

Plate Cultivations.—Shallow covered glass dishes about a decimetre in diameter

and 15 millims. deep (known as Petri's dishes) were uniformly used. These are much more simple to work with and give less contamination from the air than the original glass plate and bell-jar method. Three plates were poured for each cultivation, containing successively smaller amounts of material, so as to ensure a good plate.

In isolating organisms from sewage a second series of plates was always made to ensure perfect purity, the first apparently pure colony often covering a less conspicuous one.

PLATE 40.

PURE GELATINE PLATE CULTIVATIONS. TEMPERATURE OF INCUBATION, 20–23° C.

These figures are mainly intended as a photographic record of the relative rapidity of growth of the several organisms, and the widely differing ages of the cultures should be noted when comparing the figures; *e.g.*, fig. 6 is a plate culture five days old, whilst fig. 9 is but two days old.

Fig. 1. Anaërobic No. 2, turbid liquefied circles, 2 days old (actual size).
Fig. 2. Anaërobic No. 3, dry colony on gelatine, 7 days old, × 50.
Fig. 3. Anaërobic No. 4, filmy opalescent expansions, 2 days old (actual size).
Fig. 4. Anaërobic No. 6, non-liquefied circular colonies, 5 days old (actual size).
Fig. 5. Anaërobic No. 5, C.G.I. dark ground illumination, 3 days old, × 50.
Fig. 6. Aërobic No. 5, wax drop colonies, 5 days old (actual size).
Fig. 7A. Aërobic No. 1, non-liquefied colonies, 2 days old (actual size).
Fig. 7B. Aërobic No. 1*a*, non-liquefied colonies, 2 days old (actual size).
Fig. 8. Aërobic No. 4, non-liquefied colonies, 2 days old (actual size).
Fig. 9. Aërobic No. 3, liquefied colonies, 2 days old (actual size).
Fig. 10A. Aërobic No. 2, liquefied colonies, 24 hours old (actual size).
Fig. 10B. Aërobic No. 2, liquefied colonies, 27 hours old (actual size).

PURE PLATE CULTIVATIONS.

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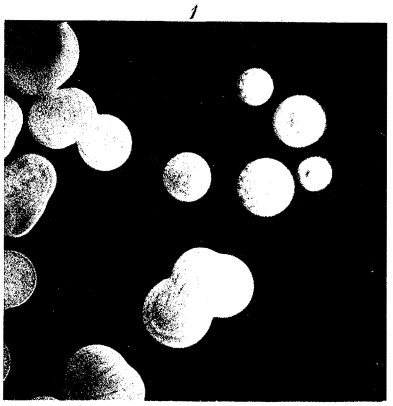

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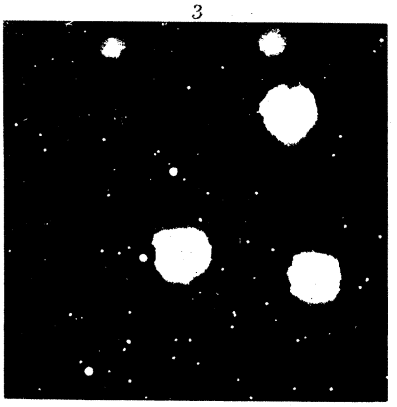

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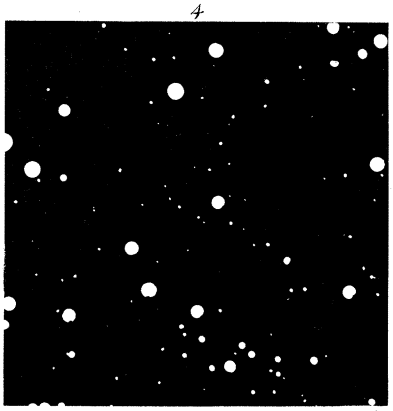

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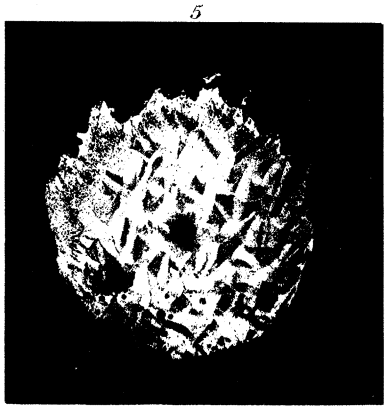

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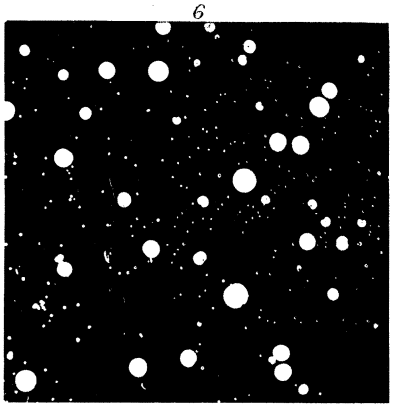

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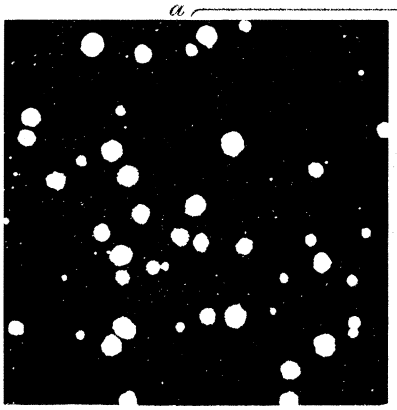

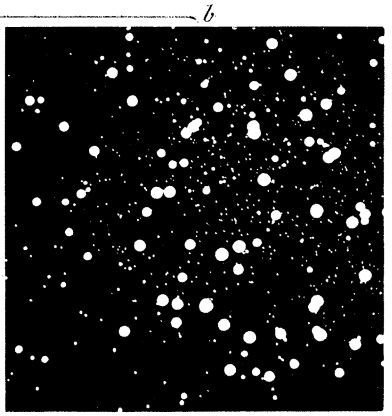

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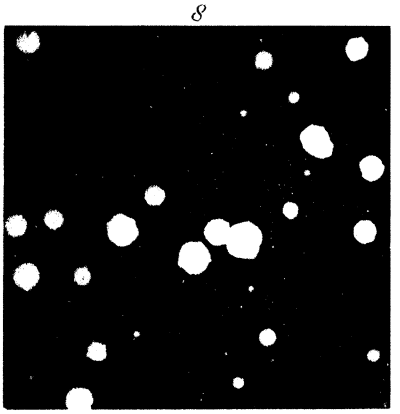

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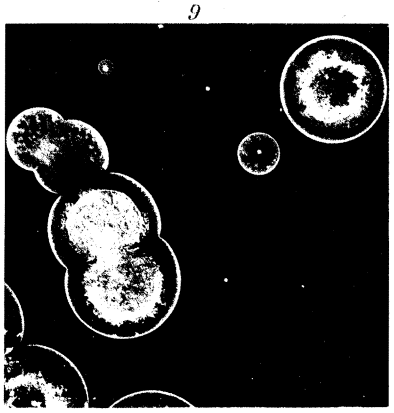

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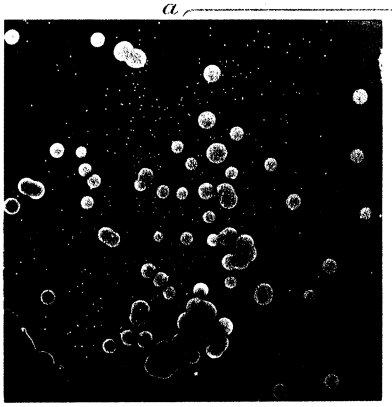

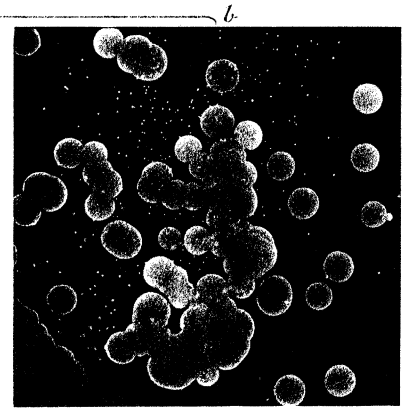

PLATE 41.

PURE GELATINE STREAK AND STAB CULTIVATIONS. TEMPERATURE OF INCUBATION, 20–23° C.

These figures not only show the relative rapidity of growth, but also the characteristic manner of growth in gelatine.

Streak Cultures on Gelatine. (All actual size.)

Fig. 1. Anaërobic No. 3, 7 days on gelatine and 7 days in broth.
Fig. 2. Anaërobic No. 4, 2 days old.
Fig. 3. Aërobic No. 4, 3 days old.
Fig. 4A. Aërobic No. 1, 3 days old.
Fig. 4B. Aërobic No. 1*a*, 3 days old.
Fig. 5. Aërobic No. 5, 3 days old.

Stab Cultures in Gelatine. (All actual size.

Fig. 6A. Anaërobic No. 1, 24 hours old.
Fig. 6B. Anaërobic No. 1, 48 hours old.
Fig. 6C. Anaërobic No. 1, 5 days old.
Fig. 7A. Anaërobic No. 2, 3 days old.
Fig. 7B. Anaërobic No. 2, 5 days old.
Fig. 7C. Anaërobic No. 2, 7 days old.
Fig. 8A. Aërobic No. 3, 24 hours old.
Fig. 8B. Aërobic No. 3, 2 days old.
Fig. 9A. Aërobic No. 6, 7 days old.
Fig. 9B. Aërobic No. 6, 14 days old.
Fig. 10A. Anaërobic No. 2, 3 days old (old culture).
Fig. 10B. Anaërobic No. 2, 3 days old (new culture
Fig. 11A. Aërobic No. 2, 24 hours old (new culture).
Fig. 11B. Aërobic No. 2, 27½ hours old (new culture).
Fig. 11C. Aërobic No. 2, 2 days old (new culture).
Fig. 11D. Aërobic No. 2, 4 days old (new culture)
Fig. 11E. Aërobic No. 2, 3 days old (old culture).
Fig. 11F. Aërobic No. 2, 7 days old (old culture).

PURE STREAK & STAB CULTIVATIONS.

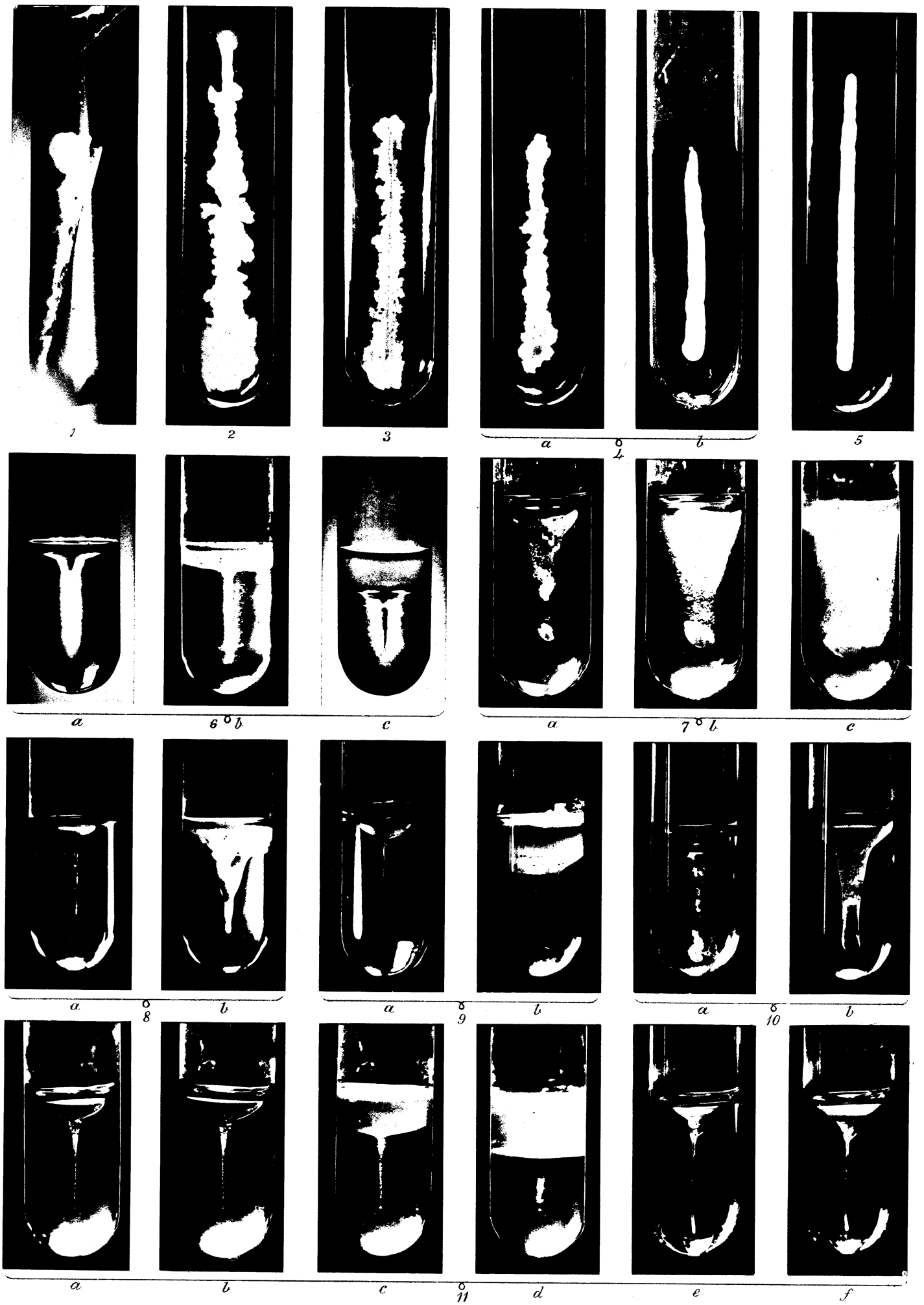

PLATE 42.

PHOTO-MICROGRAPHS.

Fig. 1. Anaërobic No. 1, cover-glass impression, gelatine plate 20 hours old, edge of colony, long rods . . . . . × 740

Fig. 2. Anaërobic No. 1, cover-glass impression showing short rods and swarmers . . . . . × 740

Fig. 3. Anaërobic No. 1, surface growth on gelatine plate, 20 hours old (from the stained plate direct) . . . . . × 100

Fig. 4. Anaërobic No. 2, short rods . . . . . × 740

Fig. 5. Anaërobic No. 2, involution forms . . . . . × 740

Fig. 6. Anaërobic No. 5, cover-glass impression, colony 3 days old, single layers of rods only fixed and stained, showing wave-like arrangement × 370

Fig. 7. Anaërobic No. 3, broth culture, 24 hours old . . . . . × 740

Fig. 8. Anaërobic No. 3, edge of colony on gelatine plate, 7 days old, nitric acid method . . . . . × 370

PHOTOMICROGRAPHS

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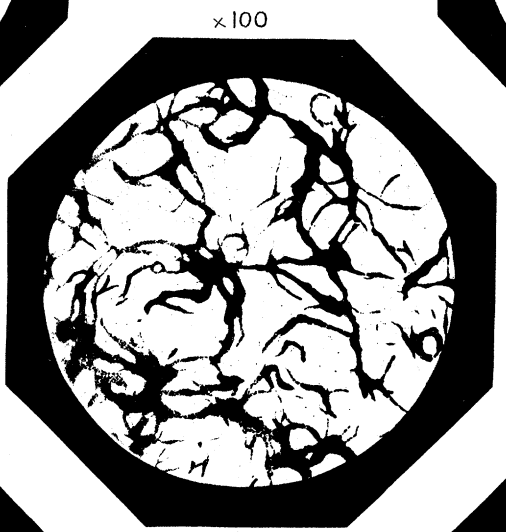

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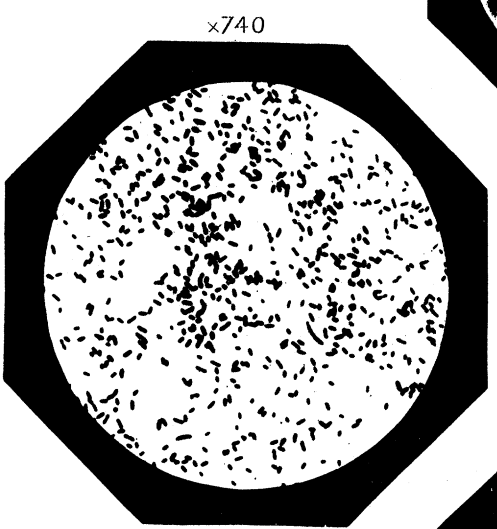

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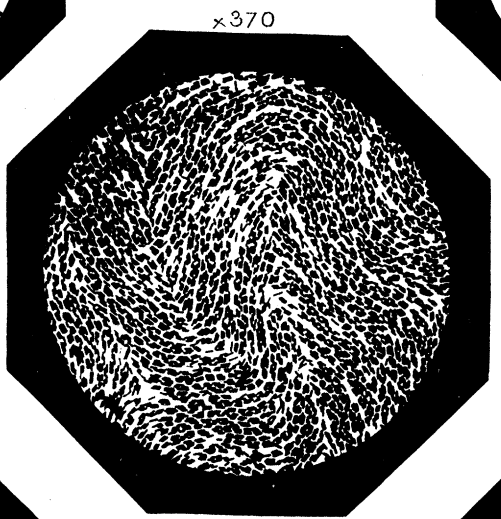

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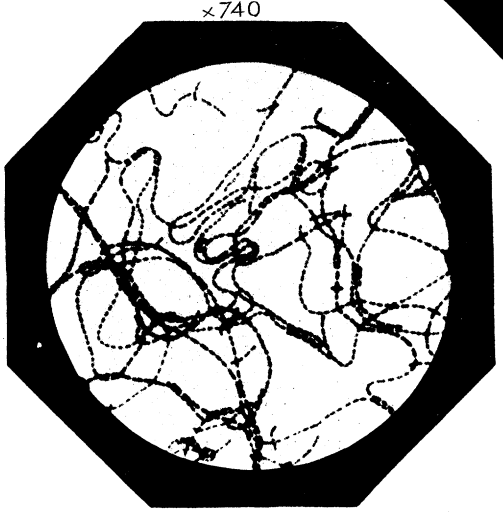

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PLATE 43.

Fig. 1. Anaërobic, No. 4, cover-glass impression, edge of colony showing long rods . . . . . × 740

Fig. 2. Anaërobic No. 4, same impression, centre of colony showing short rods and cocci . . . . . × 740

Fig. 3. Aërobic No. 1*a*, encapsuled rods, pure colony from the surface of sewage direct . . . . . × 740

Fig. 4. Aërobic No. 5, from colony on gelatine plate 4 days old, showing all the transition forms . . . . . × 740

Fig. 5. Aërobic No. 6, micrococci, from gelatine culture . . . . . × 740

Fig. 6. Aërobic No. 4, cover-glass impression, edge of colony . . . . . × 740

Fig. 7. Aërobic No. 3, cover-glass impression, surface of broth . . . . . × 740

Fig. 8. Aërobic No. 3, enlargement showing group of spore bearing rods . × 1500

PHOTOMICROGRAPHS.

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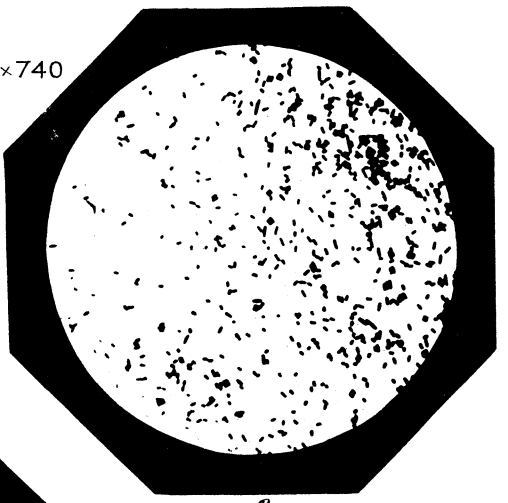

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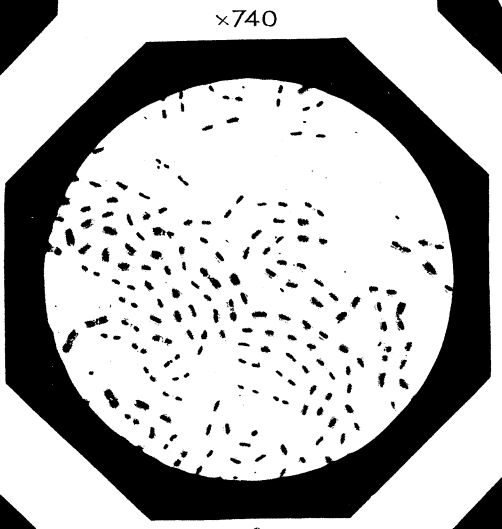

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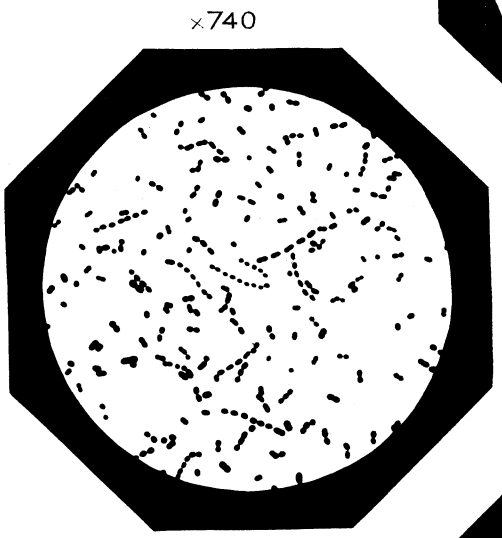

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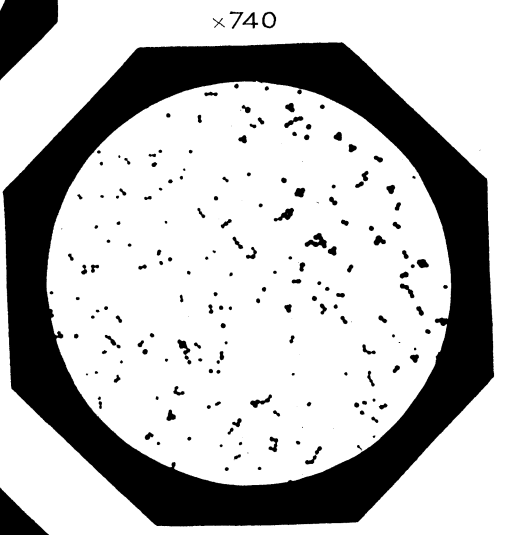

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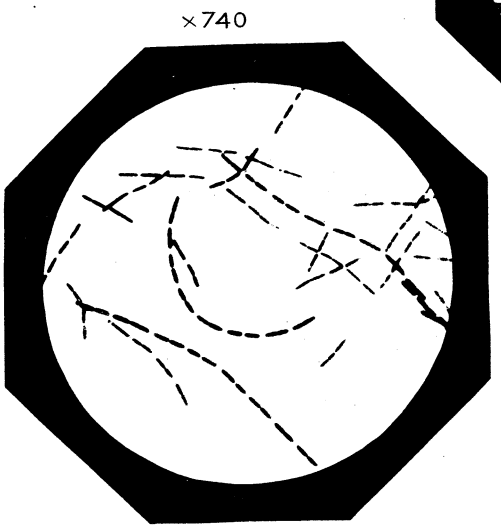

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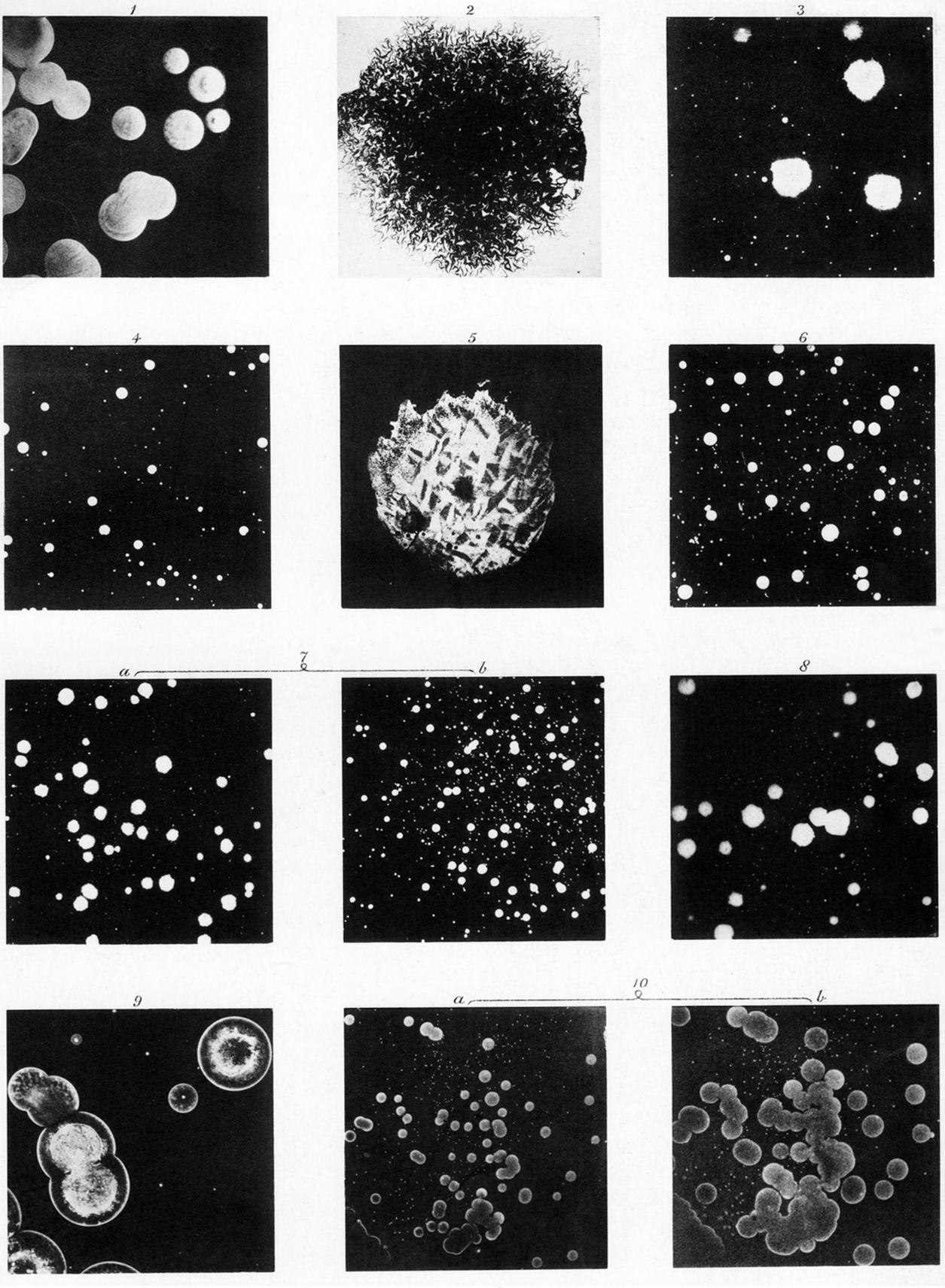

PLATE 40.

PURE GELATINE PLATE CULTIVATIONS. TEMPERATURE OF INCUBATION, 20–23° C.

These figures are mainly intended as a photographic record of the relative rapidity of growth of the several organisms, and the widely differing ages of the cultures should be noted when comparing the figures; *e.g.*, fig. 6 is a plate culture five days old, whilst fig. 9 is but two days old.

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Fig. 2. Anaërobic No. 3, dry colony on gelatine, 7 days old, × 50.
Fig. 3. Anaërobic No. 4, filmy opalescent expansions, 2 days old (actual size).
Fig. 4. Anaërobic No. 6, non-liquefied circular colonies, 5 days old (actual size).
Fig. 5. Anaërobic No. 5, C.G.I. dark ground illumination, 3 days old, × 50.
Fig. 6. Aërobic No. 5, wax drop colonies, 5 days old (actual size).
Fig. 7A. Aërobic No. 1, non-liquefied colonies, 2 days old (actual size).
Fig. 7B. Aërobic No. 1*a*, non-liquefied colonies, 2 days old (actual size).
Fig. 8. Aërobic No. 4, non-liquefied colonies, 2 days old (actual size).
Fig. 9. Aërobic No. 3, liquefied colonies, 2 days old (actual size).
Fig. 10A. Aërobic No. 2, liquefied colonies, 24 hours old (actual size).
Fig. 10B. Aërobic No. 2, liquefied colonies, 27 hours old (actual size).

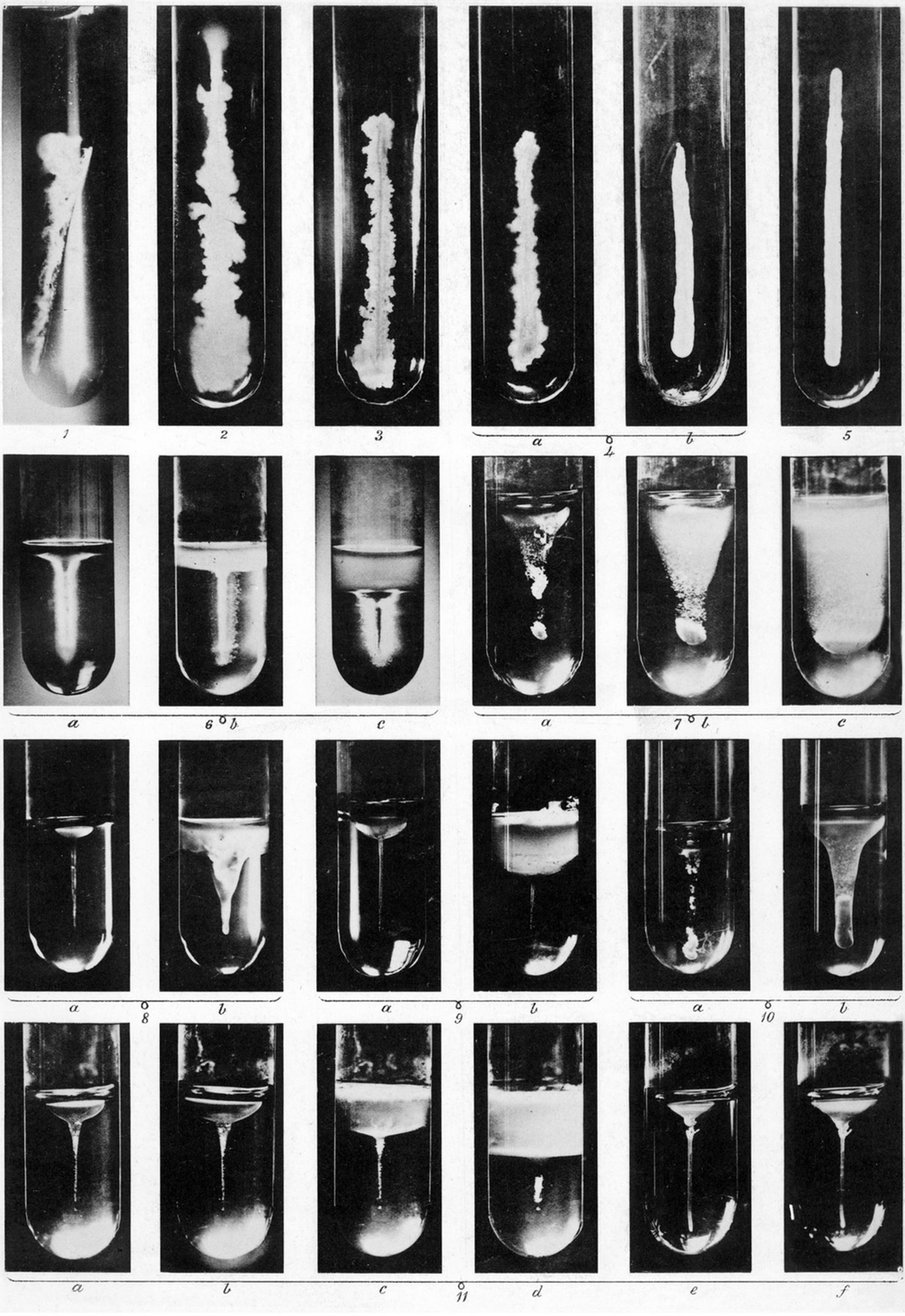

PLATE 41.

PURE GELATINE STREAK AND STAB CULTIVATIONS. TEMPERATURE OF INCUBATION, 20–23° C.

These figures not only show the relative rapidity of growth, but also the characteristic manner of growth in gelatine.

Streak Cultures on Gelatine. (All actual size.)

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Fig. 3. Aërobic No. 4, 3 days old.
Fig. 4A. Aërobic No. 1, 3 days old.
Fig. 4B. Aërobic No. 1*a*, 3 days old.
Fig. 5. Aërobic No. 5, 3 days old.

Stab Cultures in Gelatine. (All actual size.

Fig. 6A. Anaërobic No. 1, 24 hours old.
Fig. 6B. Anaërobic No. 1, 48 hours old.
Fig. 6C. Anaërobic No. 1, 5 days old.
Fig. 7A. Anaërobic No. 2, 3 days old.
Fig. 7B. Anaërobic No. 2, 5 days old.
Fig. 7C. Anaërobic No. 2, 7 days old.
Fig. 8A. Aërobic No. 3, 24 hours old.
Fig. 8B. Aërobic No. 3, 2 days old.
Fig. 9A. Aërobic No. 6, 7 days old.
Fig. 9B. Aërobic No. 6, 14 days old.
Fig. 10A. Anaërobic No. 2, 3 days old (old culture).
Fig. 10B. Anaërobic No. 2, 3 days old (new culture
Fig. 11A. Aërobic No. 2, 24 hours old (new culture).
Fig. 11B. Aërobic No. 2, 27½ hours old (new culture).
Fig. 11C. Aërobic No. 2, 2 days old (new culture).
Fig. 11D. Aërobic No. 2, 4 days old (new culture)
Fig. 11E. Aërobic No. 2, 3 days old (old culture).
Fig. 11F. Aërobic No. 2, 7 days old (old culture).

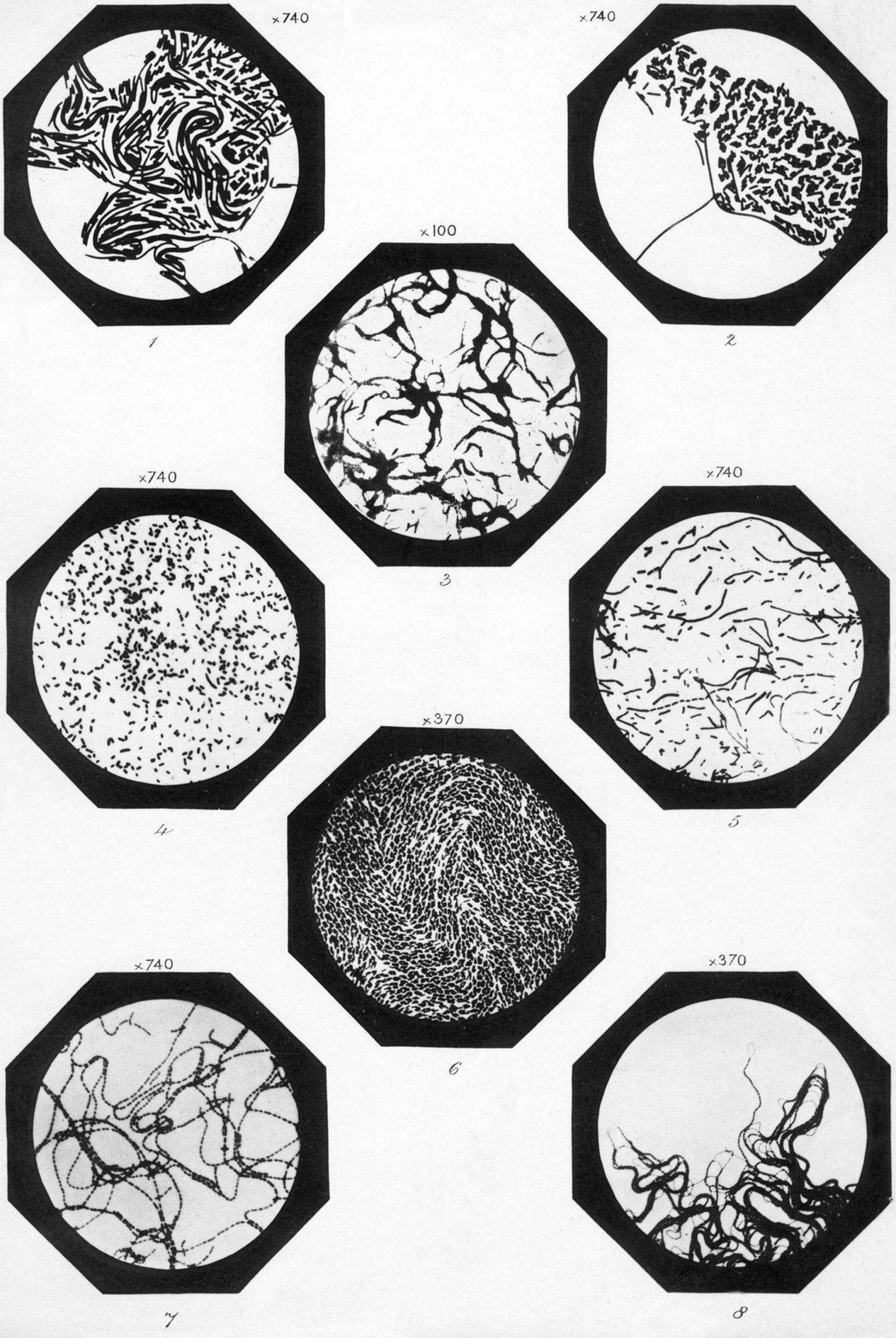

PLATE 42.

Photo-micrographs.

Fig. 1. Anaërobic No. 1, cover-glass impression, gelatine plate 20 hours old, edge of colony, long rods . . . . . × 740

Fig. 2. Anaërobic No. 1, cover-glass impression showing short rods and swarmers . . . . . × 740

Fig. 3. Anaërobic No. 1, surface growth on gelatine plate, 20 hours old (from the stained plate direct) . . . . . × 100

Fig. 4. Anaërobic No. 2, short rods . . . . . × 740

Fig. 5. Anaërobic No. 2, involution forms . . . . . × 740

Fig. 6. Anaërobic No. 5, cover-glass impression, colony 3 days old, single layers of rods only fixed and stained, showing wave-like arrangement × 370

Fig. 7. Anaërobic No. 3, broth culture, 24 hours old . . . . . × 740

Fig. 8. Anaërobic No. 3, edge of colony on gelatine plate, 7 days old, nitric acid method . . . . . × 370