

CHARACTER AND PROPERTIES OF THE "READING" BACILLUS, ON WHICH A NEW METHOD OF TREATMENT OF WOUNDS HAS BEEN BASED.¹

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(PLATE VI.)

THE following is a résumé of certain research work on which has been based a new method of treatment of septic gunshot wounds, the main outlines of which have already been published (1917⁶).

The idea gradually took shape in my mind during an investigation which I carried out as a result of a clinical observation made by one of my surgical colleagues, Major Joyce, R.A.M.C.(T.). My thanks are due to him for his kindness in affording me every facility to investigate cases under his care, for his sustained interest in the progress of the research, and for his co-operation when the time arrived to put the theories to the test. Major Joyce had been in the habit of employing the salt-pack method of treatment, originally recommended by Colonel H. M. W. Gray, C.B., A.M.S. (1915⁹) and Colonel Lawson.

His results on the whole were excellent and tallied with the published results of others who had previously employed this method. In one or two cases, however, treatment by salt-bags proved a failure, and for a time there was no explanation forthcoming, until Major Joyce made the observation that, whereas from all wounds treated successfully by the salt-bag method, a characteristic foul pungent odour was emitted, this, however, was completely absent from cases of failure. On being informed of this observation, the obvious explanation occurred to my mind that an organism or organisms were present in the one type of case, but absent from the other, and, at my colleague's request, I immediately took steps to determine if this were so. Judging from the odour emitted from successfully treated wounds, I concluded that the organism to be looked for probably belonged to the anaerobic group. Cultures were first made in cooked meat from a successful salt-bag case and grown anaerobically, with the

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result that I obtained two anaerobes growing in symbiosis. Both were spore-bearers, but while one possessed round terminal spores, the other had oval subterminal ones. The first step towards a study of the organisms was to separate them in pure culture. This I had considerable difficulty in doing until I devised the following method.

An isolated colony or part of such a colony is picked off from the solid medium with a platinum needle, and a suspension made in 1 or 2 c.c. of sterile saline. An old colony is perhaps preferable where there is practically nothing left but spores. After vigorous shaking a sample of this suspension is transferred to a hæmacytometer stage, or preferably to a counting chamber such as has been devised by Professor Ernest Glynn for the numerical estimation of bacteria in vaccines. Having ascertained the number of spores or bacilli per c.c. of the suspension, it is an easy matter to make from this a dilution which will contain approximately one spore or one bacillus per unit of saline. When the desired dilution has been obtained, the next step is to sow a series of cooked-meat tubes each with the unit volume of saline containing a single organism or spore. These are then incubated anaerobically, and thereafter examined as to their purity where growth has occurred. If a sufficiently high dilution has been made there is every probability that a pure strain or strains will be secured.

The method is simple, does not take up much time, reduces the possibility of extraneous contamination to a minimum, and, short of raising a culture from a guaranteed single bacillus, as the ideal method would be, offers a very reasonable prospect of success.

In this way each of the two anaerobes was isolated in pure culture. Only one of these emitted any odour, an odour, however, which was exactly similar to that peculiar to successful salt-bag cases. The organism responsible was the bacillus with the oval subterminal spore, and I have named it the "Reading" bacillus. From its behaviour in wounds and as a result of animal experiment it is like most spore-bearing anaerobes—a saprophyte. War-wound anaerobes have been classified by Henry (1917^{11, 12, 13}) into two great groups, saccharolytic and proteolytic, and it is in the second of these that the Reading bacillus must be placed. This grouping, while convenient, does not furnish much help when we come to identify the various members which make up these groups.

As this work was begun when the available literature on the subject of anaerobes was not only somewhat bewildering, but even contradictory, my first aim before trying to identify the organism was to isolate it in pure culture and then to study its morphological, cultural, and biological characters. These are set out in the following paragraphs.

DESCRIPTION OF ORGANISM.

Microscopic characters.—It is a rod having a fairly large oval sub-terminal spore, and is probably best seen in hanging-drop preparations where it presents a torpedo-shaped appearance, the blunter and broader end corresponding to the contained spore. From this blunt end the body gradually narrows to the opposite extremity. In dried stained

films, on the other hand, the spore appears to bulge beyond the contour of the bacillus, an effect probably due to shrinkage in the course of drying. Now and again the spore is found in the centre of a bacillus which tapers to either extremity. Very occasionally it appears to be quite terminal, but close examination will generally reveal a small tag of bacillary body projecting beyond. In young cultures non-sporing bacilli predominate. These are generally isolated, but under certain conditions may be associated with similar bacilli in the form of chains of varying length. Very occasionally in such a chain may be found a somewhat long unsegmented filament, equal in length to several bacilli. In old cultures, especially those grown on solid media, nothing may remain but free spores or spores arranged in chain fashion, sometimes with the suspicion of the outlines of what was once a filament. In cultures of intermediate age all steps in the formation of the spore can be studied.

The considerable variation in shape and arrangement, as well as the tendency to form spores, depends probably on the composition and character of the medium employed and on the length of the incubation period. The bacillus is provided with numerous long wavy lateral flagella which, when seen detached, resemble spirilla with fairly regular undulations.

The average size of the sporing bacillus varies from 2.5 to 5.3 μ , with an average breadth of 0.5 to 0.9 μ . Free spores measure on an average 1.46 μ long by 1.02 μ broad. These figures refer to the organism in the fixed and stained condition, but of course there is a considerable range of variation. Both the sporing and the non-sporing bacilli are actively motile with a sort of "waddling" movement, and this motility appears to persist for a considerable length of time.

The organism stains easily with the ordinary anilin dyes, but is not acid-fast. In young cultures it is Gram-positive. In older cultures it is less so and may even be definitely Gram-negative.

Cultural characters.—It is a strict anaerobe, but will grow quite well in a cooked-meat medium without the use of a supernatant layer of liquid paraffin and without being placed in any special anaerobic apparatus. This method of growing anaerobes is very similar to that suggested by Tarozzi (1905¹⁸). In the case of all other media, however, some form of anaerobic apparatus must be used.

Practically in all culture media, the organism develops a strong characteristic odour. It is pungent and foetid, but not unbearable when one has become accustomed to it. It is perhaps best recognised in cooked-meat cultures. If the latter have been raised from spores it does not become really strong till the third day, and in a week or ten days' time it begins to diminish in pungency.

Cooked-Meat Broth.—When inoculated from a young culture, the supernatant broth previously clear becomes, in eighteen to twenty hours, cloudy, while some of the particles of meat may turn reddish-brown in colour. In

forty to forty-eight hours the meat has begun to turn black and to diminish in volume, the particles of meat apparently going into a state of finer subdivision. Within sixty hours the volume of meat has become reduced to about half its previous bulk and presents a greyish-black colour. This gradual reduction in volume can be watched and measured from day to day, and the rate of diminution can be compared with that produced by other proteolytic ferments. During the first three days gas bubbles can be seen caught between the groups of meat particles, and the gas generated may be sufficient to force up the meat particles *en masse* so that they rest on the subjacent broth.

Where the culture to be observed has been raised from spores only, the above changes take a little longer to make themselves apparent.

If a cooked-meat culture is left standing for some considerable time, the turbidity will be found to have disappeared from the upper strata of broth, while a woolly deposit will be seen to have settled on the top of the meat. This forms a strong contrast with the sloughy remains below.

Agar.—Colonies are small, resembling in point of size those of streptococci. They are slightly raised, rounded, shiny, and at first translucent, but later tend to become more opaque. Their edges are not sharply defined, but merge into a radiating tangle of fine thread-like projections for the definition of which a hand-lens may be necessary. In place of or associated with these may sometimes be seen rather broader outgrowths resembling transparent fimbriæ.

If the water of condensation becomes infected, growth takes place fairly rapidly in the form of a faint opalescent film spreading up between the tube and the medium. Such a film frequently shows alternations of density. The agar ultimately becomes fissured and split up by the gas generated.

Deep colonies can just be seen with the naked eye after forty-eight hours' growth. Later they become slightly opaque, depending partly on their depth in the medium and partly on their size, and are of a somewhat dirty-white colour. With a hand-lens the central part does not appear to be always of a uniform shape, but may be oval, round, triangular, etc., having rather blurred edges which merge into a tangled mass of fine filaments growing out into the surrounding medium. The resulting colony looks somewhat fluffy and resembles a little speck of partially teased out cotton-wool embedded in the medium. The amount of growth and the rapidity with which it occurs depend, amongst other things, on the composition of the medium.

Blood Agar.—A copious growth is obtained in three or four days, partly confluent and partly composed of discrete colonies. The latter are small, shiny, more or less rounded flat discs with a slightly elevated node in the centre. The edge is blurred and merges into a somewhat dry, glazed-looking feathery or filamentous outgrowth, which radiates in every direction and intertwines with the filamentous processes from adjacent colonies. The colour of the confluent growth is greyish-white, in contrast to the red colour of the medium. As the culture becomes older the colour of the medium becomes brown, while the film of growth assumes a greyish-green tint in the lower part of the tube, gradually merging into a dirty-white higher up.

No digestion of the blood agar appears to take place.

Ascites Agar.—Very small but definite colonies can be seen in twenty-four hours. These resemble round, shiny domes with blurred edges, from which a tangle of spidery filaments projects. Gradually a smooth, homogeneous, shiny, opalescent film overspreads the lower part of the slope, and this comes later to resemble a thin streak of yellowish-white paint. The medium becomes fissured, but no obvious digestion occurs.

Dorset Egg Medium.—This forms an admirable medium for cultivation of the bacillus. Within twenty-four hours there is seen a moist, whitish film of growth sometimes traversed by fissures or cracks. The medium begins to turn greenish-black in colour, and at the bottom of the tube is a turbid fluid with one or two particles of medium floating in it. The culture emits a very

powerful and offensive odour. Within forty-eight hours there is marked fissuring as well as very definite crumbling away of the lowermost part of the medium. The fluid of liquefaction has increased in volume, and contains, floating in it, fragments of egg, together with oil globules. The growth on the surface of the slope becomes thicker and more creamy and resembles a layer of curdled milk. Disorganisation and disappearance of the medium take place rapidly under further incubation, till nothing is left but a blackish disgusting-looking fluid containing fragments of the solid medium.

Inspissated Horse Serum.—This medium becomes split up and begins to crumble away first at the lower part of the slope. The surface is covered with a thin film made up of closely apposed, minute pin-point colonies, almost confluent and very slightly raised. They have a somewhat dull and glazed appearance. In the course of a few days the inoculated medium appears more translucent than that in a sterile tube. Disintegration steadily progresses. If left standing undisturbed for some days a slight deposit of black pigmented material accumulates at the bottom of the tube. Over this is the turbid fluid of liquefaction and on the top a dirty-whitish flocculent layer.

Potato.—No growth visible to the naked eye has been found, even after several weeks' anaerobic culture. With a pocket lens, however, one or two moist and slightly dome-shaped colonies have been seen resembling little specks of honey.

Gelatin.—In stab culture a faint gauze-like streak appears along the needle track in thirty-six to forty-eight hours. Small globular points make their appearance, and from the needle track a few fine short lateral off-shoots appear which tend to grow outwards and upwards. Liquefaction soon commences, first at the top of the tube, where a crateriform excavation appears containing turbid fluid in which a flocculent material is suspended. The liquefaction gradually extends downwards in the form of a nipple-shaped cone projecting down into the as yet unliquefied medium. Where liquefaction has not yet taken place, the colonies are small and woolly in appearance, with a somewhat denser centre and a fringe of filaments. The end result is a turbid opalescent fluid containing woolly particles in suspension, which tend later to fall to the bottom of the tube, leaving a clear supernatant fluid.

Nutrient Broth.—Becomes turbid in two or three days, and contains numerous flocculi in suspension together with a slight woolly deposit at the bottom, which, on shaking, rises up in a cloud to become suspended in the fluid. Filaments and clostridial forms may be seen in films made from such a culture. Gradually the flocculi settle at the bottom of the tube, leaving the broth clear. The amount of gas generated, the degree of turbidity, and therefore the amount of growth appear to depend on the ingredients used and on the reaction. Glucose broth, for instance, gives a much heavier deposit than ordinary nutrient broth, and forms an excellent culture medium.

Neutral Red Broth.—In strong concentrations no change in colour is observed. In tubes containing 5 c.c. of broth, to which 0.1 c.c. of a 1 per cent. solution of neutral red has been added, growth occurs with the production of a yellowish-green fluorescence.

Fluid Egg Medium.—Growth occurs readily in this. The medium becomes a dirty-yellow colour and quite opaque. After a day or two a heavy sediment deposits, and the dirty-yellow fluid, now rather more watery, is seen to be full of small granules held in suspension. In films made from such a culture, in addition to the bacilli are seen clusters of fine radiating needle-like crystals together with much amorphous debris.

Litmus Peptone Water.—Slight growth occurs after two or three days' incubation, the tint of the litmus becoming unmistakably blue. In plain peptone water slight turbidity is seen, and if left undisturbed for a few days a scanty layer of black pigmented material will be found deposited at the bottom of the tube and, superficial to it, a second layer of greyish woolly deposit.

Under the microscope the black material appears to be amorphous, while the woolly deposit consists of bacilli and spores.

Litmus Whole Milk.—Good growth occurs in this medium, a small amount of gas is generated and a more or less typical odour produced. No clot has ever been observed even after many days' incubation. The milk becomes more granular, less opaque, and more watery. After being left undisturbed for a few days, the material in the tube is seen to be in two distinct strata, one consisting of slightly turbid watery serum, while the other is composed of a dense aggregation of minute particles. The colour of the litmus will also be seen to have become pink.

Litmus Milk with Precipitated Chalk.—Cultures in this medium yield a larger quantity of gas. The tint while not so marked as in the milk without chalk is still definitely pink.

Films made from the milk media on the fourth day show a much heavier growth of bacilli in the chalk milk than in the other. In the case of the former, large numbers of sporing and non-sporing motile bacilli are seen as well as many free spores. There are also many clumps of small irregularly shaped granules together with clusters of fine needle-like crystals. In non-chalk milk, the bacilli are in much smaller number, are mainly of the sporing type, and are only feebly motile. Filamentous forms are also seen. The granular masses are present in much larger numbers than in the chalk milk. Numerous oil globules are present in both culture media. There seems little doubt that the organism produces acid as well as gas in milk, and that this acidity tends to limit further growth of the bacillus. Where chalk has been added the growth is stronger, the milk becomes more watery, and the granular content reduced.

Sugars, Glucosides, etc.—In order to determine the purity of the strain, the reaction on these media has been observed on many occasions over a period of a year with a similar result each time. Litmus peptone water as well as litmus agar and litmus serum agar were employed, each with the appropriate amount of the test substance, *e.g.*, sugar, glucoside, etc. The peptone used was Fairchild's, while some of the sugars were not pre-war stock.

Glucose and maltose quickly become strongly acid and a considerable quantity of gas is generated. Lævulose and dextrine are also rendered acid but to a less degree, while saccharose and sorbite media respectively become lilac-tinted, indicative of a very slight acidity. Less gas is generated in the last two-named media. By means of Durham's tubes I have been able to make a relative measurement of the amount of gas generated in this class of medium.

No definite acidity has been observed in mannite, lactose, raffinose, dulcite, inulin, galactose, nutrose, starch, glycerin, or salicin, except in the last named, where, after twelve days' incubation, the blue colour was found to have altered to a lilac or slightly pinkish tint. Saccharose media, if incubated for some length of time, tends to become slightly pink, as in the case of salicin. All have a marked odour, but there are slight differences in its character in different sugars. It is difficult, however, to express in words what the odour is like.

Films made from these various media show that in glucose, maltose, lævulose, and sorbite, typical bacilli mostly of the sporing form are present in large numbers. Both short and long as well as clostridial forms are seen. Free spores are abundant, many in chain formation.

In saccharose media growth is more scanty, and there is a greater number of spores. In glycerin, mannite, raffinose, dulcite, lactose, and inulin respectively, growth is very sparse, films showing almost nothing except free oval spores.

MISCELLANEOUS GENERAL CHARACTERS.

Optimum reaction.—The optimum reaction may be stated to be +5 to phenol-phthalein. This I have determined by making up a series of nutrient broths with a range of reaction from +50 to -50 to phenol-phthalein. These reactions were determined in the cold after sterilisation had been effected.

The tubes contained equal quantities of broth, and each tube was sown with a similar and measured amount of an old broth culture which had been heated, immediately before sowing, to 80° C. for ten minutes. The tubes were incubated anaerobically at 37° C. for three days, after which they were examined macroscopically and microscopically. They were then incubated as before for a further period of five days.

The microscopic findings corroborated the macroscopic observations. Growth ceased somewhere between +16 and +10 on the one hand, and did not occur beyond -0.5 on the other.

Relation to temperature.—The range of growth lies between 18° C. and 41° C., but the best growth is obtained at 37° C. Attempts have not been made to grow it at higher temperatures.

Resistance to heat.—The spores will withstand boiling in a water-bath for fifty-five minutes, and from such, fresh cultures may be raised. This was determined as follows. Thick suspensions were made, in sterile water, of colonies grown for some weeks on agar slopes. The tube containing the resulting suspension was immersed in a water-bath kept boiling vigorously. At intervals of a minute samples of known volume were removed by means of sterile graduated pipettes and sown in tubes containing cooked meat. These were then incubated for several days and the results recorded.

Similarly, an old broth culture (original reaction = +10) was boiled over the naked Bunsen flame and measured quantities removed in the same way to cooked-meat tubes at intervals of a minute. Growth was obtained after five minutes' boiling, but not after longer exposure.

The spores therefore possess, in the particular environment tested, a high degree of resistance to heat.

Desiccation tests.—The ability of the spores to withstand drying was determined as follows. Test-tubes containing small pledgets of cotton-wool were sterilised. Into each were placed three or four drops of an old broth culture of the Reading bacillus. The tubes were then placed for forty-eight hours in a drying chamber kept at 80° C. They were then removed and stored at room temperature. At intervals of weeks sterile cooked meat and broth were added to one of the tubes, which was then incubated. In this way growth has been obtained from these spores, after having been stored in the dry condition for eight months. Exactly how long the spore remains viable

has not yet been determined, but it is already obvious that this power to remain viable for months in the dried condition might be made use of when considering a suitable means of distribution of the organism for purposes of wound treatment. As a vehicle for the transmission of spores, sphagnum moss seems to be peculiarly suitable. The moss contained in small bags forms an ideal anaerobic packing for wounds, as has already been indicated in a Report to the Medical Research Committee. The countless microscopic chambers which it contains are easily impregnated with bacilli and their spores by growing the bacillus in broth containing moss. The latter when dried would thus not only be available as an anaerobic dressing, but would also serve the purpose of conveying the bacillus to the wound.

Growth in relation to salt.—In the course of this research I had formed the opinion that the value of the salt-bag dressings did not depend on the salt contained therein, but on the fact that this type of dressing rendered the wound sufficiently anaerobic for the Reading bacillus to be able to start active growth. This I was able to prove by the substitution of sphagnum moss for salt. In order, however, to determine whether the presence of salt was directly beneficial to the growth of the organism or not, I made certain observations on the behaviour of the Reading bacillus in the presence of various concentrations of salt. The method employed was to put up two series of tubes, one containing ordinary nutrient broth, the other glucose broth in quantities of 5 c.c. each, in which a known quantity of salt had been dissolved. In each series there was a range of salt concentration from 0 to 25 per cent. To each was added a measured quantity of an old culture of the Reading bacillus grown in cooked meat, and the tubes were incubated at 37° C. for eight days anaerobically. Growth was found to be strongest in the broth to which no salt had been added, but it had also taken place in the various concentrations up to 5 per cent., the numbers of bacilli becoming less and less as the concentration increased. In tubes with a concentration of 6 per cent. salt, growth was very feeble, films made from such showing almost nothing but spores. In higher concentrations no growth was observed. The spores, however, were not all dead, since subcultures made from the tubes in which growth had not taken place yielded in some instances a typical growth on incubation. This holds good of subcultures made from various concentrations of salt up to 15 per cent. Beyond this limit, subcultures remained sterile. The Reading bacillus, therefore, does not proliferate actively, *in vitro* at least, where salt is present in greater concentration than 5 per cent., while the growth is less in 5 per cent. than in lower concentrations. This seems to point to the fact that high concentrations of salt in the wounds are not only unnecessary, but are detrimental to the growth of the Reading bacillus if this concentration were maintained.

Fortunately for those who use salt-bags this initial concentration very rapidly diminishes.

Growth in relation to certain antiseptics.—Acriflavine, phenol, mercuric iodide, eusol, and Dakin's solution were chosen. They were put up in varying proportion each in 10 c.c. of broth, to which measured quantities of a broth culture containing spores of the bacillus were added. The inoculated tubes were then incubated anaerobically at 37° C. The cultures were examined macroscopically and microscopically, and the type of bacillus as well as the relative number of spores noted.

In the case of phenol and mercuric iodide the limiting concentration lay between 0 per cent. and 1 per cent. of a 1 in 20 solution, a typical growth being obtained in the former concentration. In the case of acriflavine, growth of the Reading bacillus occurred up to a certain point of concentration, beyond which the organism failed to grow. By subculturing afresh, however, from the highest concentration in which growth had occurred to the next higher in which no growth had previously been obtained, a culture could now be raised. In this way, starting with a culture growing in broth containing 0·2 per cent. of a 1 in 1000 dilution, the organism could eventually be made to grow in 1·6 per cent. of a 1 in 1000 dilution. This suggested that a strain of the bacillus could be evolved which would become increasingly resistant to the antiseptic. I found, however, that there was a fallacy in these experiments, in that the numbers of organism sown were not identical in each case. Five sets of culture tubes accordingly were put up. Each set consisted of ten tubes containing nutrient broth, in which 1 in 1000 acriflavine had been dissolved, so that the concentrations of antiseptic ranged from 0·1 per cent. of this solution to 1 per cent. A suspension was then made in sterile saline from young agar-slope cultures of the Reading bacillus and the number of organisms per c.c. counted. An equal volume of bacillary suspension was delivered to each of the fifty tubes, but whereas the first ten received 250 millions of bacilli each, the second series received 87 millions, and so in diminishing numbers till the fifth series received only 6 millions. The cultures were then incubated anaerobically for twenty-one days, weekly observations being made. As a result it was found that the growth in any given concentration of acriflavine appears to depend on the number of organisms sown. In other words, stronger concentrations of acriflavine must be employed up to a certain point according as the number of organisms sown is increased. Subcultures made from the acriflavine broths in which no growth had taken place showed that the spores had not been killed by a 1 in 100,000 solution of acriflavine after twenty-one days' exposure.

In the case of eusol and Dakin's solution, growth of the Reading bacillus was obtained after twelve days' incubation in broth containing

26 per cent. of eusol, while growth only took place in concentrations of Dakin's solution up to 22 per cent. Owing to a precipitate being thrown down by the addition to ordinary broth of eusol in higher concentrations, gelatin, glycerin broth, glucose peptone water, and inulin broth were tried. Growth was obtained after some days in all in presence of the highest concentration of eusol tested, namely, 36 per cent. This is what we might expect, having regard to the instability of these two antiseptics. Negative results may be obtained if the cultures are only examined after a comparatively short period of incubation, but, if incubated longer, growth eventually takes place. Dakin's solution appeared to have a slightly greater inhibitory effect than eusol. While it does not follow that the same degree of inhibition would be shown by the antiseptic when applied to the organism in the wound, these experiments furnish some information as to the resistive power of the Reading bacillus to various concentrations of antiseptic in a certain environment. They further indicate that, so far as acriflavine is concerned, the inhibitory effect depends on the concentration of antiseptic used and on the mass infection present. In the case of antiseptics like eusol and Dakin's solution other factors come into play into which I need not enter here.

PATHOGENICITY.

A large number of rabbits, guinea-pigs, and mice have now been inoculated; cultures grown in various media and for varying lengths of time have been injected both intravenously, subcutaneously, intramuscularly, and intraperitoneally without producing any bad results either local or general. No gas gangrene has ever been produced, and in no case has death supervened. For details of these experiments reference must be made to my original thesis.

The Reading bacillus has now been extensively introduced into wounds in horses and mules by Captain Dalling, A.V.C., and others, without producing any untoward result, while in Reading and elsewhere it has been freely sown in military wounds, including the knee and various other joints, and always with the best results. Captain Dalling's report will be published in due course. This organism, then, can definitely be said to be absolutely non-pathogenic in pure culture to animals. In human beings and in horses, even in the presence of all sorts of pathogenic organisms, its introduction into wounds has never been followed by any pathogenic effect, but on the contrary has invariably led to a rapid cleansing of the wounds.

CELL RESPONSE TO INJECTIONS OF THE READING BACILLUS.

Two rabbits were inoculated with 6 c.c. each of a three-day culture in meat broth, one intravenously and the other intramuscularly.

Leucocyte counts were made for three days before and for seven days after inoculation. Counts made a few hours after inoculation showed a definite fall in the number of leucocytes. This was soon followed by a definite leucocytosis, more marked in the case of the animal inoculated intravenously.

A thick suspension of young agar-slope cultures was next made in sterile normal saline.

A series of mice were inoculated intraperitoneally with 0.5 c.c. of this suspension. These mice were then killed at varying intervals, one every hour or two during a period of forty-three hours. The peritoneal cavity was immediately opened in each case and films made therefrom. In ten hours practically no organisms could be found in the films. Whereas at the end of the first hour lymphocytes preponderated, these were rapidly replaced by a polymorpho-nuclear leucocytosis, the cells appearing actively phagocytic towards the bacilli and their spores. In about ten hours after inoculation the number of polymorphs had begun to wane and large mononucleated cells to make their appearance. These in turn showed themselves actively phagocytic towards the polymorphs still present. At the end of forty-three hours the polymorphs had almost reached their initial number, while the majority of the remaining cells consisted of lymphocytes and large mononuclears in practically equal numbers.

Instead, therefore, of proliferating in the living peritoneal cavity, the organisms appear to be rapidly attacked by the polymorphs which gradually ingest them, while many of the polymorphs are themselves disposed of by large mononuclear cells. There is thus evidence that the bacillus exerts a chemiotactic influence on the polymorpho-nuclear leucocyte, and that the organism does not thrive in healthy living tissues. It is interesting to note on reference to their paper that Dean and Mouat (1916⁶) have found that the organism they describe as *B. oedematis maligni* is readily taken up by the polymorphs, judging from film preparations made from wound discharges.

RELATION OF READING BACILLUS TO OTHER SYMBIOTIC ORGANISMS.

A series of observations were made to determine if the Reading bacillus in any way influenced the numbers of other organisms growing in symbiosis with it. This was done, largely with a view to proving or disproving the existence of any inhibitory effect exercised by the Reading bacillus, either as a result of substances produced by it or as a result of its more active growth.

The organisms employed were *Staphylococcus aureus*, *Streptococcus longus*, *B. coli*, *B. pyocyaneus*, *B. typhosus*, *B. paratyphosus* (B), *B. dysenteriae* (Shiga), and *B. dysenteriae* (Flexner). Each of these was inoculated into six meat-broth tubes. Three of each set of six were reserved as controls. To each of the others was added a

measured quantity of a culture containing the Reading bacillus. Two cultures of each organism, one with the Reading bacillus and one without were incubated anaerobically. A similar set were incubated without being put up anaerobically, and a third set were incubated aerobically and anaerobically on alternate days. Every two days a measured quantity of each was plated out, the plates so inoculated being incubated aerobically and the resulting colonies counted.

The results seemed to indicate that, with the possible exception of *B. typhosus*, no pronounced inhibitory effect is produced by the growth of the Reading bacillus growing in symbiosis with any of the above-named organisms. Even in the case of the possible exception, the difference is not sufficiently pronounced to enable me to say that the Reading bacillus had any effect on the growth of *B. typhosus*. These findings are partially corroborated by observations which I had previously made on the numbers of organisms present in wounds before and after treatment with salt-bags. Pathogenic organisms are neither crowded out, nor is their further growth inhibited by the symbiotic presence of the Reading bacillus, so that the explanation of the beneficial action of the latter organism must be looked for in another direction. No evidence is forthcoming from these observations that the Reading bacillus possesses any bacteriolytic property. No attempt was made in the above experiments to estimate the relative numbers of the Reading bacillus present in these various cultures after symbiotic growth. Films made at various times from the above cultures showed, however, that the Reading bacillus was apparently not present in the same amount in all, although it had definitely grown in every culture.

SUBSTANCES PRODUCED BY THE GROWTH OF THE READING BACILLUS IN COOKED-MEAT MEDIUM.

Large quantities of gas are liberated in the course of its attack on the protein, etc., in this medium. These consist for the most part of carbonic acid gas, ammonia, and hydrogen sulphide. The latter, probably in the presence of iron derived from constituents in the culture medium, is sufficient to account for the blackening of the meat. All three gases are easily collected and demonstrated. Skatol is also produced as a result of its proteolytic action, but I have not been able to demonstrate the presence of indol or of mercaptanes.

Proteins are finally split up into amino-acids, and in certain of the media, egg, for example, many bundles of fine needle-like crystals are formed suggestive of the presence of tyrosin.

The most important vital function of the Reading bacillus appears to be a proteolytic one, the result of an enzyme or enzymes, probably of the nature of proteases, and it is these latter that furnish the key

to the *modus operandi* of the organism when used as a means of treatment.

Proteoclastic properties are also possessed by filtrates from broth cultures of the organism, so that to a certain extent the enzyme or enzymes may be said to behave as an ectocellular product and can thus be isolated for purposes of study. The filtrate, however, is not quite so rapidly proteolytic as is the living culture. Comparative observations have been made of the action of trypsin, Reading bacillus culture, and filtrates from the latter on gelatin, coagulated egg albumin, cooked meat, and casein respectively. As a result of these tests it has been found that the enzymic action of the Reading bacillus bears a close resemblance to that of trypsin, while the end results of hydrolysis are much the same in each case. For instance, into each of a series of tubes containing sterile distilled water, maintained at boiling-point, was dropped 1 c.c. of egg albumin. When cool, the water was pipetted off the coagulum. To each of a number of such tubes were added 5 c.c. of various dilutions of trypsin in 1 per cent. Na_2CO_3 , the dilutions ranging from 1 in 50 to 1 in 10,000. To another tube, containing the coagulated egg albumin, were added 10 c.c. of Reading bacillus filtrate, while to yet another were added 5 c.c. sterile saline, which was then inoculated with the Reading bacillus. The tubes were incubated at 37°C . In the case of the filtrate the egg albumin had disappeared in four and a half days, the same result being achieved in the same time by trypsin in a dilution of 1 in 1500. The egg albumin in saline had, under the influence of the living Reading bacillus, entirely disappeared in sixty hours.

As with trypsin, there comes a time when the accumulated products of hydrolysis lead to a slowing down and finally to a cessation of the reaction, but this occurs more quickly with trypsin than with the Reading bacillus. Treatment of wounds by the latter possesses numerous and obvious advantages over any method of treatment with an animal ferment. Artificial gastric juice has, it appears (1915¹), actually been used for treatment purposes.

There is growing evidence (1916^{2,3}, 1918¹⁶) that eusol and Dakin's solution which are so successful in wound treatment depend not on any direct antiseptic property they may possess, but on their strong proteolytic properties. They are able to play the part of inorganic catalysts and to hydrolyse the necrotic material in the wound. Support is lent to this view by a paper (1916¹⁴) published on the intravenous use of eusol, and again by observations (1916⁴) carried out on the destruction by eusol of the toxin of *B. dysenteriae* (Shiga). The use of Dakin's solution or of eusol is open to one great objection, however, namely, that as their power depends on a bulk chemical action the amount to be used must bear a direct ratio to the amount of protein destroyed.

The Reading bacillus, on the other hand, is a permanent manu-

factory of a proteoclastic enzyme whose initial velocity will be more or less maintained throughout owing to constant removal by the wound discharges of the products of its hydrolysis.

While most bacteria possess the power of attacking protein, only a few have the power of forming proteases in any appreciable amount, and probably still fewer possess the power of hydrolysing the protein substrate in such a way that the destruction products are themselves non-toxic.

That the Reading bacillus appears to belong to this select group seems conclusively proved both by clinical and by experimental observations made in the course of this investigation. While it acts as an organic catalyst, the Reading bacillus is able, moreover, not only to hydrolyse the necrotic tissue in a wound, but it appears to be equally capable of rendering non-toxic the degradation products of other pathogenic organisms. In other words, it is apparently able, while actively engaged in the removal of necrotic tissue, the breeding-ground of pathogenic organisms, to modify the amount of toxic absorption by the patient. This line of thought was suggested by the fact that if the improvement which follows the introduction of the Reading bacillus depended merely on hydrolysis of the necrotic tissue, it ought not to take place till all the sloughs have disappeared. As a matter of fact, however, it occurs much sooner, and it was therefore necessary to find an explanation. Supposing that the toxins, the degradation products of pathogenic bacteria, were protein in nature, it followed that these toxins might share the same fate as the necrotic tissue undergoing proteolysis by the bacillary enzyme.

ACTION ON TOXINS.

To throw some light on this possible explanation I resolved to expose a given toxin to the action of the Reading bacillus by growing the latter in broth containing some of that toxin. For purposes of demonstration I required a toxin of high potency, capable of provoking definite clinical signs, able to produce its effects in small doses (thus eliminating the danger of using massive quantities), which at the same time could be accurately measured and inoculated in graduated quantities for purposes of comparison. Tetanus toxin seemed to fulfil these conditions, and this I chose to work with in the first instance. Fluid tetanus toxin and desiccated toxin were kindly placed at my disposal, the former by Dr. A. T. MacConkey, the latter by Dr. O'Brien, to both of whom I am deeply indebted. Commencing with the first type of toxin, I found it very definitely modified in the direction I had hoped, by the growth in it of the Reading bacillus. Owing to technical difficulties, however, I abandoned it for the desiccated form, which I found much more stable. The experiments were conducted over a period of several months, and certain of them were repeated on several

occasions. Guinea-pigs of known weight were used throughout the experiments, of which the technique was as follows:—

A known quantity of the desiccated toxin was accurately weighed out when required for the experiment, observing due precaution to prevent gross error arising from absorption of moisture by the hygroscopic powder. This amount was then dissolved in a previously sterilised solution of 0·85 per cent. sodium chloride in water. From this, measured amounts were pipetted off and delivered into known volumes of sterile broth so as to obtain dilutions of toxin of known strength.

Sterile nutrient broth was used, the reaction of which was for some experiments +10, for others +5. Some of these were then inoculated with the organism whose effect I wished to observe, while others were left uninoculated to serve as controls. The whole of the tubes, inoculated and uninoculated in any one experiment, were then incubated anaerobically at 37° C. for so many days.

At the end of the incubation period the contents of each inoculated broth tube were filtered through separate porcelain filters. The contents of the uninoculated tubes were treated in the same way, so that all would be subjected to the same manipulation. In order to obtain the necessary range of doses for inoculation, each filtrate was further diluted with suitable amounts of sterile sodium chloride solution (0·85 per cent.). Each animal to be inoculated was carefully weighed, and the required dose injected into the muscles of the thigh. The animals were thereafter kept under close observation, the time noted when signs of tetanus were first observed, and the date of death recorded when a fatal issue ensued. A wide range of doses was employed, the smallest being well below the actual M.L.D. of the desiccated toxin which, by the way, remained approximately the same throughout the whole of the experiments.

In the same way the effect of growing certain other organisms in broth containing tetanus toxin was observed. These organisms were *B. perfringens*, *Vibrio septique* (Amatzi), *B. sporogenes* (Metchnikoff), and *B. histolyticus*. In all about seventy animals were inoculated.

The results showed that a guinea-pig could stand as much as 148 times the M.L.D. of tetanus toxin in which the Reading bacillus had previously been grown without any effect other than some stiffness of the inoculated limb, which soon recovered. Doses corresponding to 80 times the M.L.D. and lower never developed even local tetanus.

Animals inoculated with samples of tetanus toxin, in which other organisms had been grown, developed typical tetanus, and died even where the dose employed was not more than a single M.L.D. There was one exception, however, namely, that in which *B. sporogenes* had been grown. Such a toxin appeared to be so modified that the animal was able to withstand doses larger than the M.L.D. without developing tetanus, but the modification was not nearly so marked as in the case of tetanus toxin in which the Reading bacillus had been grown.

It is a remarkable thing that such an organism as *B. histolyticus*, which has a much more powerful proteolytic action than the Reading bacillus, should nevertheless be unable to split up the toxin of tetanus. So powerful are its proteolytic properties, so far as dead tissues are concerned, that it constitutes one of the best agents for rapidly macerating bones, and one that ought to be of some value to those who

prepare museum specimens. I have used it for this purpose with success.

Having demonstrated by repeated animal experiment that the Reading bacillus possessed the power to modify tetanus toxin so profoundly that an animal could withstand almost 150 times the M.L.D., I desired to know if other toxins could be affected in the same way. To this end I employed diphtheria toxin and also a filtrate from cultures of *B. perfringens* grown in cooked-meat broth. For the former I am again indebted to Dr. A. T. MacConkey. The technique was as before, except that the only organism whose effect on the toxin was studied was the Reading bacillus. Controls were again kept and subjected to the same conditions as the toxin in which the organism had been sown. After incubation, and inoculation into guinea-pigs, it was found that at least sixty times the M.L.D. of diphtheria toxin in which the Reading bacillus had been previously grown was followed by no ill effect whatever, whereas the controls died.

In the case of the *perfringens* filtrate, in which Reading bacillus was subsequently grown, the dose of pure toxin necessary to kill was found to be rather massive, so that no great range of doses could be studied. This is an objection to which I have already referred. The material was injected into the peritoneal cavity of guinea-pigs. A very slightly larger dose of *perfringens* filtrate, in which Reading bacillus had been grown, was followed by no ill effect, whereas the control animal died in five days. An animal inoculated with a similar dose in which *B. histolyticus* had been grown also died, but more rapidly than the control.

In this way I was able to demonstrate the power of a proteolytic organism to reduce the toxicity of certain toxins, whereas the other organisms investigated, with the possible exception of *B. sporogenes*, were unable to effect a similar change. In view of the close resemblance of the bacillary enzyme to trypsin, I was curious to know whether the latter would produce the same effect on tetanus toxin. The procedure was much as before except that after dissolving the measured amount of desiccated toxin in sterile saline solution, appropriate quantities of the latter were transferred to tubes containing 1 in 50 trypsin in 1 per cent. hydrated Na_2CO_3 dissolved in sterile distilled water. The amount of tetanus toxin in each c.c. was thus known. The tubes were incubated at 37° C. anaerobically, and in the dark, so as to obviate modification of the tetanus toxin by these factors. As a result it was found that trypsin likewise possesses the power of detoxicating tetanus toxin. A sufficient range of doses, however, was not studied, so that I cannot say whether trypsin is equally potent in its power to modify this toxin. For details of these experiments reference must be made to my thesis.

Sufficient has been said, however, to show the importance of the biological properties of the Reading bacillus, not only in relation to

the dead tissue, but to certain toxins as well. It is instructive to remember that, in the hands of Dean and Adamson (1916⁴), eusol was able to modify the toxic element of Shiga's bacillus. The above experiments are further of interest from the point of view of determining the nature of bacillary toxins.

The question of hydrolysis of the toxic substrate, however, leads one into the sphere of colloid chemistry and involves us in problems which only the biochemist is competent to handle.

QUESTION OF IDENTITY OF THE READING BACILLUS.

For reasons already given, I deferred making any attempt to identify the Reading bacillus with any known type until I had been able to study it more minutely. It was obvious, however, that, if the organism had not already been described, its frequency in salt-packed wounds at least was certain to have been noted. The organism must obviously be placed in the proteolytic group of anaerobes, and as such probably belongs to what Miss Robertson (1916¹⁷) in an extremely valuable paper calls Group D, which embraces *B. œdematis maligni* (Koch), *B. cadaveris sporogenes*, *B. tetani*, and *B. botulinus*. From the two latter it is easily differentiated on the grounds of its non-toxic properties, apart altogether from other points of difference. From the two former there is greater difficulty in distinguishing it. On grounds of morphology it would seem as if the Reading bacillus is distinct from *B. cadaveris sporogenes*, as referred to by Miss Robertson. There is one left, namely, *B. œdematis maligni* (Koch), and on the possible identity of this with the Reading bacillus it is more difficult to express an opinion. There are two points of difference, however. One of them is of minor importance, namely, the reaction produced in milk; the other is of greater moment, namely, the entire lack of pathogenicity on the part of the Reading bacillus. As some strains of Koch's bacillus appear to be pathogenic while others are not, I prefer in the meantime, till the significance of these differences has been fully understood, to adhere to the name "Reading bacillus" for the strain which I have isolated. The name *B. œdematis maligni* has too sinister a connotation, and it appears, moreover, to be indiscriminately applied to more than one type of organism. Other bacteriologists have referred more or less vaguely to an organism which may be similar to the Reading bacillus. For instance, Fleming (1915⁷), working at Boulogne, refers to two anaerobes which he designates X and Y respectively. They are stated by him to be responsible for the foul smell of gangrenous wounds, and are said to be non-pathogenic. Judging from illustrations, the bacillus X seems to resemble the Reading bacillus morphologically, but again there is a want of data by which to make a comparison. Goadby (1916⁸) gives a brief description of the characters of an organism recovered from war wounds which he

identifies as *B. oedematis maligni*. His description, so far as it goes, seems to coincide with that of the Reading bacillus. The fact that his organism may have round or oval spores rather raises a suspicion that he is not dealing with a pure strain. Dean and Mouat (1916⁵), on the other hand, describe an organism which seems to correspond in all essential points, so far again as their description goes, with that of the Reading bacillus. Their organism appears to be non-pathogenic to animals. They consider that its presence in the wound is not necessarily of serious import, that, in short, its activities are confined mainly to the dead tissues, and that it has little or no capacity for multiplication in the living tissues. Further identification stops here. They seem to take it for granted that anaerobes in general produce, by their ferment action, poisonous substances, possibly organic acids, which progressively damage the living tissues. That one at least of these anaerobes does not follow this general rule appears proved by the clinical and experimental work carried out on the Reading bacillus, and shows that it is not safe to make too hasty generalisations. J. E. G. Harris, working at a certain laboratory in France, was kind enough to interest himself in the biochemical properties of the Reading bacillus, and ventured the opinion that it bore a close resemblance to *B. sporogenes* (Metchnikoff). The result of his extremely careful and laborious comparative experiments will, I understand, be published shortly, and I therefore refrain from making further allusion to them. He was kind enough to send me subcultures of the strain of *B. sporogenes*, with which he was working, so that I might compare it with the Reading bacillus. Morphologically and culturally the two organisms are strikingly similar. They both give the same sugar reactions, and behave generally in a similar manner in various other media. There are one or two minor points of difference. For instance, colonies grown on agar remain more compact than those of Reading bacillus, which tend quickly to become surrounded with a fringe of fine filaments. This is not invariably the case, but on the whole the colonies of the Reading organism appear to develop these filamentous off-shoots to a greater extent than do colonies of *B. sporogenes*. Then again there is a close resemblance between the two organisms in relation to their effect on tetanus toxin. *B. sporogenes*, I have mentioned, is the only one of the anaerobes investigated that shows any power of splitting up the toxin. This power, however, does not appear to be so marked as in the case of the Reading bacillus, since animals will stand a larger dose of tetanus toxin in which Reading bacillus has been grown than of toxin modified by *B. sporogenes*.

Finally, through the kindness of M. Weinberg of Paris, who sent me some *sporogenes*-agglutinating serum, I was able to test the latter's power of agglutinating the Reading bacillus, and to compare the effect produced on that organism with the effect on *B. sporogenes*. At

the same time, the ability of normal serum to agglutinate either of these organisms was investigated, while simple suspensions of the organisms in saline served as further controls. The technique was as follows:—

Measured quantities of suitable dilutions of the sporogenes-agglutinating serum were delivered into small tubes. To each series were added similar amounts of a saline suspension of young agar-slope cultures of the Reading bacillus and *B. sporogenes* (Metchnikoff) respectively, so that the ultimate dilution of agglutinating serum ranged from 1 in 50 to 1 in 3200. The dilutions of normal serum used were 1 in 50 and 1 in 100. The tubes were placed in the water-bath at 55° C. for two hours, and the results read at the end of sixteen hours. They were as follows. No agglutination occurred in the simple saline suspensions, and none in the tubes containing normal serum in the dilutions used. *B. sporogenes* (Metchnikoff), of which three different sub-cultures were tested, was agglutinated up to a dilution of 1 in 800, in which, however, the reaction was slight, indicating that the limit of agglutination had been reached. In the case of the Reading bacillus, agglutination was still almost complete in a dilution of 1 in 3200. Hence we are faced with this extraordinary fact, that a serum in which agglutinins had been formed for *B. sporogenes* (Metchnikoff) possessed a stronger power of agglutination over the Reading bacillus. The significance of this would take too long to discuss here, and I must content myself with recording the fact.

The behaviour of the Reading bacillus in the presence of this agglutinating serum supports the view that it is closely related to but not necessarily identical with the *B. sporogenes* (Metchnikoff), and this conclusion harmonises with other observations to which reference has already been made. It rather goes to support Henry's (1917¹²) view that "it is not unlikely that one is dealing with a whole group of bacilli rather than with an individual." He goes on to say that while cultural features have so far failed to serve as a basis of differentiation of the various members, serum reactions may do so. That this view is probably a correct one, the above findings tend to prove. To the serum reactions I would add an entirely new test, namely, the reaction of the organism in relation to such a toxin as that of tetanus—a test that will probably be of some value in dealing with the proteolytic group.

Metchnikoff (1908¹⁵) himself considered that there were two types of *B. sporogenes*, one of which he isolated from healthy fæces, the other from diarrhoeic stools. As a matter of interest I took the trouble to examine some of the specimens of fæces sent in from convalescent dysenteric patients. Some thirty specimens in all were investigated, and these were derived from twenty-four patients. All yielded negative results, except one received from a patient, the subject of ankylostomiasis. The bacillus recovered from this man's fæces showed all the morphological and cultural characters of the Reading bacillus. It seems rather a small percentage from which to recover the organism, and it does not suggest the human intestinal tract as a common habitat of this organism. My own view is that it

will be found to be a common inhabitant of the soil, especially where decaying protein matter is present.

My thanks are due to my assistants, Miss A. B. Clark, B.Sc., and R. H. McLean, D.Sc., for their ungrudging help, to Miss Muriel Robertson for certain strains of organisms for purposes of comparison, and to the Medical Research Committee for financial assistance in procuring animals for purposes of experiment.

SUMMARY AND CONCLUSION.

1. The above work, on which I have based a new method of treatment for gun-shot wounds, was undertaken in order to find an explanation for a certain clinical observation made by a surgical colleague.

2. The method depends on the utilisation of the properties of a certain organism whose morphological and cultural characters are here described, together with a method of isolating such organisms from mixed culture.

3. The bacillus is a spore-bearing anaerobe of a saprophytic nature, belongs to the proteolytic group of anaerobic organisms, and is probably present in the majority of gunshot wounds, but its activities are generally held in abeyance by the system of wound-dressing usually adopted. I have named it the Reading bacillus for reasons already given. It is highly resistant to heat and drying, and grows best in a slightly alkaline medium.

4. It most closely resembles *B. sporogenes* (Metchnikoff), from which, however, it differs in certain points. Perhaps it would be correct to say that it is probably one particular strain in what may be called the *sporogenes* group.

5. Experiments show that it is non-pathogenic for animals as well as for man when introduced in the latter into septic wounds. It does not attack living tissues, the cell response of which to the introduction of the bacillus has been investigated.

6. Its behaviour in relation to salt and to certain antiseptics is described. The former is not necessary for the successful treatment of gun-shot wounds, as was thought by those who advocated the salt-bag method. The success depends rather on the activity of this particular bacillus under conditions favourable to its growth and not on the salt.

7. The rationale of the method depends not on inhibition by the Reading bacillus of the growth of pathogenic organisms in the wound, either by reason of the formation by the bacillus of any inhibitory organic acid, or by the production of any bacteriolytic ferment, as symbiotic experiments show.

8. It acts, however, by virtue of its proteoclastic enzymes as an organic catalyst which hydrolyses the substrate of dead protein. It

disintegrates the protein base from which pathogenic organisms operate, and while so doing does not itself give rise to fresh toxic substances.

9. Not only so, but it is probably able to hydrolyse also the toxic degradation products of other organisms. In support of this a résumé is given of experiments on tetanus and other toxins, which show that the Reading bacillus, out of a series of organisms investigated, is alone able to reduce the toxicity of these toxins. There is one exception, namely, *B. sporogenes* (Metchnikoff), which, however, does not appear to be so potent in this direction as is the Reading bacillus. The ability to disintegrate necrotic tissue does not necessarily imply an equal power of hydrolysing toxins, as is illustrated by the experiments with *B. histolyticus*, which may be highly useful for museum work but would probably be dangerous in wounds.

10. This ability to modify a toxin like that of tetanus may prove to be of value as a means of differentiating various types of proteolytic organisms, while it introduces new ideas in regard to the biological processes going on in septic gunshot wounds. To treat a septic wound successfully involves a knowledge of how and what toxins are produced by the pathogenic organisms present, and the crude attempt to sterilise a wound by endeavouring to kill off the organisms by the simple application of antiseptics must in time give way to a method based on a more intimate knowledge of the biological processes at work. One is inclined to think that too much time has already been wasted in what appears to be a vain endeavour to find an ideal antiseptic.

This method which I have called the biological method is, I venture to hope, a step in this direction, and it is one that is intimately bound up with questions of colloid chemistry, further work on which may lead to other important therapeutic developments.

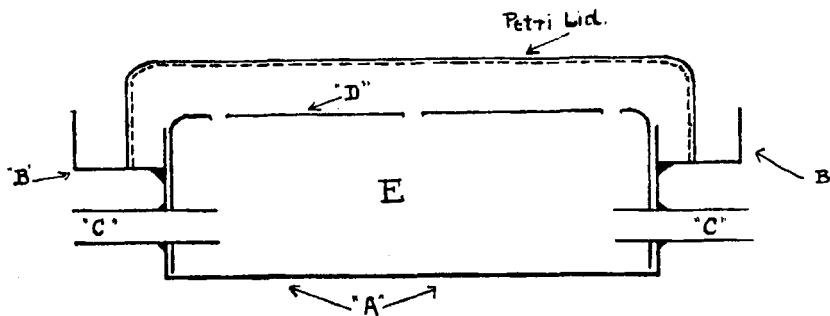
APPENDIX.

1. **For Tube Culture.**—Where large numbers of anaerobic cultures have to be investigated at one time, the following method which I have devised for use here may be recommended.

Empty 2-lb. Glaxo tins are used. As these are of convenient shape, less cumbrous, unbreakable, and will hold about sixty tubes at one time, they are superior to the ordinary Bulloch's apparatus. They can very easily be adapted for laboratory use as follows: A film of solder is run along the seams between the bottom and the body of the tin, as well as along the vertical seam on one side of the body. The lid of a Glaxo tin is flanged, the flange fitting tightly down on a ledge, the former projecting slightly over a shallow trough running round the circumference of the tin external to the lid. By a simple device this lid can be rendered air-tight. All that is necessary when the lid is *in situ*, is to pour a little melted paraffin wax into the trough. On solidifying, the tin will be found to be hermetically sealed. It is necessary to be able to fill the tin with hydrogen or to introduce pyrogallate of soda. This is achieved in the following way. Two holes are punched through the lid and two right-angled metal tubes are inserted into the holes thus made, so that one of the tubes projects some distance below the lid, and therefore into the tin. To

this downward projecting arm is attached a piece of pressure tubing, long enough to reach nearly to the bottom of the tin. To the right-angled limbs projecting outside are fitted two pieces of pressure tubing provided with clips. By connecting up to a Kipp's apparatus, the available oxygen may be replaced by hydrogen, while, if required, after creating a slight vacuum, pyrogallate of soda, prepared as recommended by Haldane (1912¹⁰), may be introduced.

2. For Plate Culture.—The apparatus consists of three pieces. The lowermost, "A," resembles an inverted tin lid made in one piece, to the outer side of whose rim has been soldered a right-angled ledge of tin, "B." This runs all the way round and, together with part of the vertical rim of "A," forms a trough or fossa. The breadth of this fossa is one-eighth of an inch, and its depth measured on the inner side is a quarter of an inch. The diameter of the tin tray is three inches, and its depth three-quarters of an inch. Piercing the side of tray "A" are two small tin tubes, "C," which project slightly into the interior of the tray and outwards for half an inch. Attached to each of these is a small piece of pressure tubing controlled by a clip. These tubes allow of the entry of hydrogen or of pyrogallate of soda. The second piece of tin, "D," again resembles a lid in the uninverted position. It is made of such a size that it fits fairly closely into tray "A." It is provided with two right-angled slits in its rim so that when pushed home into tray "A" the slits slip



over the projecting inner ends of the two tubes, "C." By giving "D" a slight turn, a bayonet catch action comes into play, so that "D" becomes firmly fixed in position and will not fall out when the apparatus is turned upside down. When "A" and "D" are in position they enclose a chamber, "E," seven-eighths of an inch deep in which pyrogallate of soda may be contained. The top of lid, "D," is pierced by a series of small holes, to allow of gaseous communication between the chamber and the culture. An improvement kindly suggested to me by Professor Sims Woodhead would be the insertion of a vertical partition across tin, "D," so dividing the chamber, "E," into two portions. In this way one would be certain of filling the whole apparatus with hydrogen, for, in order to get from one side of the partition to the other, it would have to traverse the space lying between the glass cover and the top of tin, "D," *via* the holes in the latter. The top of tin, "D," may be japanned to act as a dark background, against which colonies may be more readily seen. The third part consists of one of the two glass plates of an ordinary Petri dish. Before use some cotton-wool is placed in chamber, "E," and the whole apparatus fitted together is sterilised in one piece. For use the apparatus is inverted, so that the glass plate is lowermost, the melted and inoculated medium rapidly poured into it, and the tin part, which acts exactly like the other half of a Petri dish, replaced. When the medium is set the apparatus is reversed, so that the glass part is now uppermost, and a small quantity of melted paraffin wax is now run into the trough, so that, when it solidifies, the glass lid will be found firmly fixed to the tin ledge and quite air-tight. The further technique

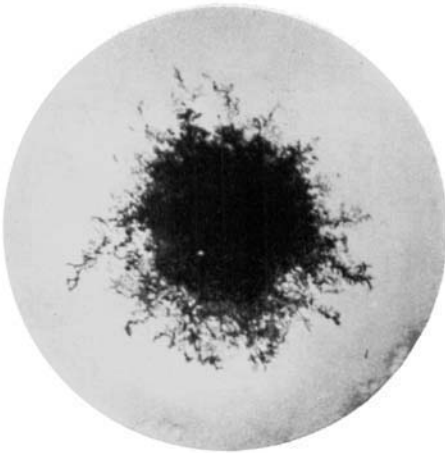


FIG. 1.

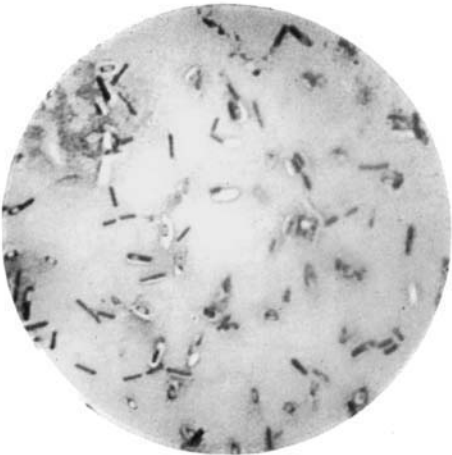


FIG. 2.



FIG. 3.

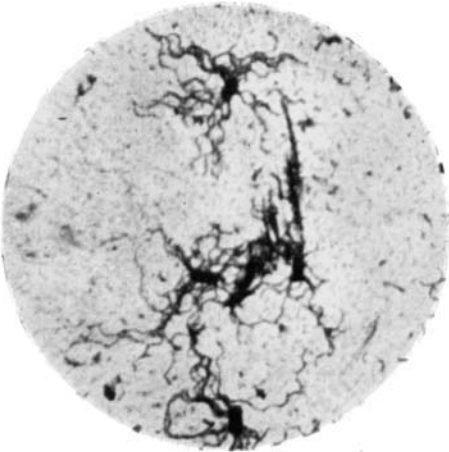


FIG. 4.

consists in displacing the available oxygen by driving in hydrogen, or, if required, pyrogallate of soda may be introduced. The vertical depth of the whole apparatus need only measure one and a quarter inch. The ledge is made of such a width that various sizes of glass lid may be used. It possesses certain advantages over such an apparatus as M'Leod's or Henry's (1917¹³) in that the whole apparatus can be sterilised in one piece. The risk of air-borne contamination is reduced to a minimum, as the lid is only opened for a second for the introduction of the medium. It can be hermetically sealed in a few seconds more easily than by a method of plasticine luting, and it allows of the use either of hydrogen or of pyrogallate of soda. The apparatus is cheap, and is made for me by Huntly, Bourne, & Stevens, Reading.

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DESCRIPTION OF PLATE VI.

FIG. 1.—Colony from agar culture, eighteen days old. ($\times 10$.)

FIG. 2.—Reading bacillus and spores. ($\times 1000$.)

FIG. 3.—Reading bacillus showing oval subterminal spores. ($\times 1500$.)

FIG. 4.—Reading bacillus showing flagella. ($\times 1500$)