AN INVESTIGATION OF THE CULTURAL REACTIONS OF CERTAIN ANAEROBES FOUND IN WOUNDS.¹

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(PLATES XXVI.-XXXIV.)

FROM the time of the discovery of Pasteur's *Vibrio septique* onwards, the investigation of the anaerobe has stimulated the energies of a host of workers, so that a very large volume of literature on the subject is available at the present moment. Much of the work was done at an early period in the history of bacteriology, at a time, that is, when the more precise technical methods of the present day were non-existent, and it is for this reason of limited value. But even now there is considerable confusion in regard to the characteristics of the better known species, so that it is often difficult to pick out from the literature a concise and reliable account of any one individual organism.

The contradictory statements which beset the literature dealing with anaerobes may arise from two reasons: firstly, individual species may show marked variations from time to time in their cultural reactions; or, secondly, the difficulties inherent to anaerobic technique may result in the investigation of cultures which are not pure. As our work with the anaerobes has progressed, we have come to assign the burden of responsibility in this matter of discordant statements more and more to impurity of culture, and we are convinced that some at least of the anaerobes show no more variation than do certain well-established aerobic organisms.

The work herein detailed was undertaken with a view to determining the rôle played by anaerobes in infected tissues, and it has resolved itself into several stages. At the outset it became necessary to devise a ready means of isolating anaerobes in pure culture. The technique of the method ultimately adopted to secure this result has been elsewhere described by Henry (1917¹⁴), and is referred to here only in such details as appear to be essential.

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The second stage in the investigation has consisted in the recording of the cultural features of pure growths on a variety of media, and with this there has resulted an aggregation into groups of individuals presenting common characteristics. The media used have been such as are already in use among British workers for the study of anaerobes, and can be easily prepared in any laboratory. Only those organisms have been dealt with which appear to conform to types already described by other investigators. We have divided the anaerobes into two main groups, the saccharolytic and the proteolytic, and we believe that a recognition of such a classification, founded as it is on the basis of biochemical activities, is of considerable service in establishing the rôle played by anaerobes in the tissues. The anaerobe differs from the aerobe in that it exhibits in the presence of organic material active catabolic changes many of which are capable of analysis by chemical methods.

The present communication is divided into the following sections :—

- I. The isolation of anaerobes in pure culture.
- II. The morphology and cultural features of the more important anaerobes found in wounds.
- III. The differentiation of anaerobes by means of sugar reactions.
- IV. The changes produced by anaerobes in the tissues.

I.

THE ISOLATION OF ANAEROBES IN PURE CULTURE.

The term anaerobe was used by Pasteur to designate an organism which could not develop in the presence of air or of free oxygen. To accept the term in its strictly literal sense, and to define an anaerobe as a microbe which is capable of reproducing itself without the aid of oxygen is to deny to this particular type of organism one of the essentials of protoplasmic activity. The anaerobe requires oxygen for its development just as much as does the aerobe, but it is able to make use of oxygen only under special circumstances. Such anaerobes as have been isolated from wounds exhibit, each and all, vigorous fermentation reactions in the presence of organic matter, and it would seem to be precisely by reason of this power that this group of organisms is able to make use of oxygen in combination in preference to free oxygen. The growth of anaerobes under apparently aerobic conditions, by the addition of pieces of animal or vegetable tissue to the culture medium, methods described by Tarozzi (1905³¹), Wrzosek (1907⁴²), and others, is no more than the presentation to the organism of oxygen in an accessible form.

This matter of the utilisation of combined as opposed to free

oxygen is one of the fundamental differences between the anaerobe and the aerobe. But there are other distinctions of equal importance, which must be borne in mind. The ordinary simple laboratory media which give such constant results with most aerobic organisms are but ill-adapted for anaerobic work, and to obtain success it becomes necessary to make use of media rich in organic material, either of a protein or carbohydrate nature. Further, the reaction of the media is of paramount importance, for probably it is only within a definite range of reaction that the ferments elaborated by any individual species of anaerobe can exert their power and so enable the organism to make use of the foodstuff present.

The essentials then for anaerobic growth consist in—

1. The provision of oxygen in an available form.
2. The presence of organic material which can be broken down by ferment action.
3. The adjustment of the contents of a medium to such a reaction as will facilitate and accelerate the fermentative activities of the organism.

The establishment and maintenance of this trinity of essentials can be effected in a variety of ways, and the modern worker has at his command a large number of media and a variety of devices which will give successful results. The problem, however, that confronts one is not to get an anaerobe merely to grow, but to get it to grow under such conditions that it may be readily isolated from the organisms with which it is associated. The separation of an aerobe in pure culture by picking off isolated colonies from the surface of some suitable solid medium is a relatively easy operation, but the process is beset with certain difficulties in the case of the anaerobe. An emulsion of anaerobes planted out under the best possible conditions for growth may give but a poor harvest in colonies. It is difficult often to obtain a growth proportionate to the seeding, such as one expects in the case of the aerobes. This peculiarity militates against purity of cultures, for in picking off an apparently pure colony one may transfer with it organisms which are of a different character and which have not developed because conditions were not suitable for their growth. Hence it follows that an apparently pure culture of one anaerobic micro-organism may give fixed and regular reactions through a series of subcultures and yet prove in the end to be mixed.

No one has more ably insisted on the necessity of a rigorous technique in the isolation of anaerobes than has Miss Muriel Robertson (1916²⁵).

The anaerobes isolated from wounds may be divided into two main groups on the basis of their biochemical reactions.

I. **The Saccharolytic Group** contains a number of individual species which give fermentation reactions in the presence of various

carbohydrates but which show little tendency to attack proteins; it comprises—

1. *B. welchii* (Welch and Nuttall).
2. *B. tertius* (The *B. von Hübner* ix. or *B. rodella* iii. of British writers).
3. *B. fallax* (Weinberg).
4. *B. aerofetidus* (Weinberg).
5. *B. oedematiens* (Weinberg).

II. The Proteolytic Group.—The characteristic of the members of this group is the vigorous digestive action they develop towards protein material, while their carbohydrate splitting power is of limited range. The group contains—

1. *B. sporogenes* (Metchnikoff).
2. *B. histolyticus* (Weinberg).
3. *B. putrificus coli* (Bienstock).
4. *B. tetani* (Nikolaier).

There is no hard-and-fast boundary line between these two groups. Certain strains of *B. welchii*, for instance, do produce amino-acids in small quantities from protein, and *B. aerofetidus* reduces protein still further so as to give sulphuretted hydrogen. Both, however, are essentially saccharolytic rather than proteolytic in their action on organic matter, especially when they are compared in this respect with members of the second group.

As a general rule, the individuals of Group I. develop very rapidly in the presence of a fermentable carbohydrate, producing acid and gas in considerable quantity, whereas the individuals of the second group, the proteolytic group, grow more slowly in cultures.

In view of these biochemical distinctions it becomes impossible to devise any one single method that is applicable to the separation of anaerobes in general, for a medium which favours the growth of a carbohydrate-splitting organism may be quite unsuitable for the development of a protein digester. The method used by most French workers for the isolation of anaerobes is that originally devised by Veillon and Zuber (1898³⁴), and consists in planting out an increasing scale of dilutions of the infected material, with or without previous heating, into a series of deep glucose-agar tubes while these are still in the liquid condition. The seeded cultures are then incubated, and out of those tubes, which show sufficiently discrete growth, isolated colonies are picked out and planted into fresh tubes. Some anaerobes, however, do not grow rapidly in the presence of glucose, while others which show good growth may produce so much gas that the agar becomes fragmented and, so, useless for the purpose of isolation of individual colonies. In addition to these disadvantages the method involves much labour and demands an amount of time that is denied to most workers.

Again, the resistance of spores to heat forms the basis of a method

by which these may be segregated off from vegetative forms, but certain anaerobes form spores only with difficulty, so that the method became one of limited utility. Attempts to isolate the spores of one species of anaerobe from those of another have not so far given constant and reliable results, although Von Hibler (1908¹⁵) claimed it to be a valuable means of separation.

The measures we have found to be most useful in the preparation of pure cultures of anaerobes consist in repeated plating alternating with growth in selective media. The separation of spores by heating may be used when opportunity occurs as an adjuvant to this procedure. The following scheme which deals with the method in more detail has been found useful in the isolation of the organisms indicated:

B. welchii.—Infected material presumably rich in *B. welchii* and reasonably free from contamination may be planted out directly on to plates. Muscle juice or subcutaneous oedema fluid, also heart blood or visceral blood obtained at post-mortem, may all be treated in this way with a reasonable measure of success. Where there is much admixture with other microbes, as in a wound discharge, the material is first seeded into alkaline minced meat or into milk before plating out. The presence of glucose in the first medium and of lactose in the second, both sugars that are vigorously fermented by *B. welchii*, give the organism the opportunity of developing so rapidly that it soon outnumbers all the other microbes present. This numerical preponderance, however, is in evidence only in the earlier stages of growth, for in the later stages the development of more slowly growing organisms, and the acid production that results from fermentation, together help to reverse the position, so that *B. welchii* in a viable state occurs in rapidly decreasing numbers. In the case of meat cultures *B. welchii* attains its maximal development in from three to six hours, and it is thus possible to pass the same material through two meat tubes between the morning and evening of the same day.

The second culture may be plated out in the evening and left to grow overnight. Colonies picked off next morning may again be passed through meat tubes, and the process can be repeated as often as is necessary. This method of intensive culture, which results in the passage of *B. welchii* through two meat tubes and one agar plate in twenty-four hours, has given very good results in our hands. In most cases a growth which is practically certain to be pure can be obtained in six days, *i.e.*, after six agar platings and twelve rapid passages through alkaline meat. In certain cases, however, it may be necessary to carry on the process for two or three days longer. To ensure purity the strains we have used for the sugar reactions have each been plated out eight times and put through alkaline meat sixteen times after the initial plating.

If the original material contains *B. welchii* in spore form, the elimination of all vegetative microbes by a preliminary heating to 80° for twenty minutes will much facilitate the task of isolation.

The alkaline meat may be replaced by a broth or casein medium containing 1 per cent. maltose or glucose. For some time we used, for the purpose of intensive culture, 1 per cent. glucose chalk broth to which a section of fresh sterile grape was added, the medium being left quite open to the air and without a layer of superimposed paraffin oil. This was no more than a modification of the Tarozzi method and gave just as good results as the alkaline meat but possessed no outstanding advantages over the latter. Maltose is more rapidly and more vigorously fermented than glucose, and with a 1 per cent. maltose broth, under paraffin and freshly sterilised, it is possible to make from four to

six replants in the course of a day. The value of alkaline meat, however, over the other sugar-containing media, lies in that *B. welchii* lives in it for long periods; so that, if for any reason the work of isolation be interrupted, the culture can be put on one side and kept for later investigation.

The device used for anaerobic plate culture throughout our work has been already described (Henry, 1917¹⁴). To get good luxuriant growths of *B. welchii* it is necessary to seed from young meat cultures four to six hours old, and to smear the surface of the agar at the time of the inoculation with alkaline egg albumin. This is best done by mixing equal volumes of the egg albumin and of the meat culture in a Pasteur pipette, expelling a drop of the mixture on to the agar plate, and using the pipette as a spreader after bending it in the flame. Pure strains of *B. welchii* may be kept for long periods of time in alkaline egg albumin, in which the organism spores with great constancy. Stock cultures kept in this medium for six months have been found to give vigorous sub-cultures when planted again into meat, and it is probable that the organism is capable of living in alkaline egg even much longer.

B. sporogenes.—After *B. welchii* this has been found to be the most frequent microbe in wounds. In contradistinction to *B. welchii* it develops relatively slowly in cultures, and it spores readily in all media. The routine method we have employed for isolating *B. sporogenes* is as follows. Suspected material, tissue or exudate, is inoculated into milk and incubated for five days. A portion of this milk culture is then heated to 80° for twenty minutes and planted into alkaline meat. The heating eliminates all the organisms which do not spore in milk, including *B. welchii*, only the spores being transferred to the meat medium. If *B. sporogenes* be present there is vigorous digestion of the meat with blackening and the development of a foul odour. After five days' incubation the meat culture is plated out on to agar. Fluffy colonies resembling those given by *B. sporogenes* are picked off the plate after three days' incubation and planted into meat again. A second planting is done after three days in meat, and the process of alternating three days on agar with three days in meat is repeated until a pure growth is obtained. As a rule, three plantings after the first combined with three passages through meat are sufficient to get a pure culture.

The mixture of *B. welchii* with *B. sporogenes* which one finds so frequently in muscle juice and in post-mortem heart blood or portal blood can frequently be dealt with by direct plating on to agar, the subsequent steps in purification being similar to those above indicated.

Or again, such a mixture after heating may be sown into alkaline egg and planted out on to agar after five days' incubation. The implantation into alkaline egg gives the proteolytic *B. sporogenes* the chance of outgrowing *B. welchii*.

B. tertius.—This organism is probably identical with that described by Fleming (1915⁸) under the name *Bacillus Y* and also with that described by Miss Robertson (1916²⁵) as *B. von Hübner* ix. or *B. rodella* iii.

It is frequently met with on agar plates during the process of isolation of both *B. welchii* and *B. sporogenes*, and gives colonies which are so characteristic as to be readily recognised after a little experience. It forms spores with constancy in liquid media, and may easily be isolated by following the same routine as that adopted in the case of *B. sporogenes*. Infected material is maintained in milk for five days. The culture is then heated to 80° for twenty minutes and planted into meat. The development of gas and acid in this meat after twenty-four to thirty-six hours is very strongly suggestive of the growth of *B. tertius*. A portion of this meat culture, two days old, is planted on to agar and after two days' incubation on agar isolated colonies are passed into meat again for twenty-four hours. The process of passage on to agar for two days and through meat for one day is repeated several times, and with care four or five such passages are enough to give a pure culture.

A much simpler method for the isolation of *B. tertius* consists in the use of broth or of casein containing either mannite or salicin, both of which are readily fermented by this organism (see Sugar Reactions). Passage through mannite or salicin for twenty-four hours alternating with two days' incubation on agar, if repeated three times, affords a very reliable method of getting this organism in pure culture.

These three organisms, namely, *B. welchii*, *B. sporogenes*, and *B. tertius* are the most frequent found in wounds, their relative frequency being indicated in Table I.

TABLE I.—To show the Incidence of certain Anaerobes in Pathological Material.

Material Examined.	Total Examined.	Total Septic.	Septic Cases infected with—			
			Anaerobes.	<i>B. welchii</i> .	<i>B. sporogenes</i> .	<i>B. tertius</i> .
1. Open wounds, two to twenty-two days old.	100	100	72 = 72 per cent.	66 = 66 per cent.	48 = 48 per cent.	22 = 22 per cent.
2. Hæmothorax fluids, <i>i.e.</i> , closed wounds of pleura and lung.	500	195	87 = 44·6 per cent.	71 = 36·4 per cent.	14 = 7·1 per cent.	1 = 0·5 per cent.
3. Post-mortem heart blood one to twenty hours after death.	40	36	25 = 69·4 per cent.	20 = 55·5 per cent.	15 = 41·6 per cent.	1 = 2·7 per cent.

During the Somme fighting a systematic cultural investigation was made at a Base Hospital in France of pus obtained from 100 open wounds of varying degrees of severity, and taken at an interval of from two to twenty-two days after the receipt of the injury. In all these cases the cultures were carried sufficiently far to establish a bacteriological diagnosis as to the presence or the absence of the three organisms in question, while in certain instances the microbes present were ultimately isolated in pure cultures and used for further study. About two-thirds of these wounds contained *B. welchii*, about one-half showed *B. sporogenes*, and about a quarter contained *B. tertius*. In practically all cases the anaerobes are mixed with aerobes, and as a rule more than one anaerobe is found to be present, a very frequent combination being *B. welchii* with *B. sporogenes*. It is by no means easy to decide how far these findings give an indication of the organisms carried into a wound at the time of or immediately after the injury, for it is certain that the flora which gains entrance at the beginning subsequently undergoes very considerable changes. In

contradistinction, however, to these open wounds we have had the opportunity of examining a series of hæmothorax fluids resulting from penetrating wounds of the pleura and lung. These may be looked upon as closed wounds and differ from the open septic wounds in two important particulars. In the first place, the anaerobes which develop in the pleural sac are those which have been implanted at the time of injury. There is no question of a subsequent addition of anaerobes. And, secondly, the anaerobes in the pleural sac have less chance of making headway, for they have to develop in defibrinated blood, whereas in the open wound they are frequently implanted into or come in contact with damaged muscle.

Out of 500 specimens of hæmothorax fluid examined, 195 proved to be septic, and of these septic cases 45 per cent. were found to be infected with anaerobes. Here again *B. welchii* is the most common infecting anaerobe, and the combination of this organism with *B. sporogenes* is frequent.

The third series of figures in the table concerns itself with the presence of anaerobes in the blood stream after death. Out of forty cases from which death resulted from wounds, which were proved to be infected with anaerobes, the heart blood taken from one to twenty hours after death gave positive cultural evidence of anaerobic invasion on twenty-five occasions. In twenty of these *B. welchii* was found, and in fifteen *B. sporogenes* was present. The question of the appearance of the anaerobe in the circulating blood will be dealt with later.

The other anaerobes dealt with in this paper are those which have been described during the war by Weinberg. It is probable that we have isolated *B. oedematiens*, but we have not yet been able to identify it among our cultures; and *B. histolyticus* we have seen only in mixed cultures.

B. fallax was certainly confused in the earlier stages of our work with *B. welchii*, and it was only after reading Weinberg's description and examining a strain of this organism kindly sent by him from Paris that we have come to recognise it and to look for it in cultures. After some search we have obtained four strains of *B. fallax*, each having been found in the course of isolating *B. welchii* from muscle juice, *i.e.*, by plating out after rapid passage through meat.

We have had exactly the same experience with *B. aerofetidus*, for, after investigating a strain sent by M. Weinberg, we found among our cultures two organisms which presented similar characteristics. Since then we have collected five strains in all, one from post-mortem heart blood, one from a hæmothorax fluid, and three from muscle juice. In each case, as with *B. fallax*, the organism has been found during the isolation of *B. welchii*.

II.

THE MORPHOLOGY AND CULTURAL FEATURES OF THE
MORE IMPORTANT ANAEROBES FOUND IN WOUNDS.**B. welchii.***A. The Identity and Relationship of B. welchii.*

B. welchii was first fully described by Welch and Nuttall in 1892 ⁽⁴⁰⁾ under the name of *B. aerogenes capsulatus*. It may be briefly described as a large Gram-positive non-motile anaerobic bacillus, which rapidly produces stormy fermentation when grown in milk. Its proteolytic power is slight, but it is strongly saccharolytic, producing butyric acid from the sugars it ferments. This particular feature of the biochemical activities of *B. welchii*, i.e., the production of butyric acid, is common to a number of anaerobes, and has been taken by various workers as a criterion of value in the classification of these organisms. Simonds (1915 ²⁹), for example, in his monograph on *B. welchii*, follows the lead of others in classifying *B. putrificus coli* (Bienstock) and *B. oedematis maligni* (Koch) among the butyric-acid-producing organisms; but the strongly proteolytic power of these two organisms is in itself sufficient to exclude them from close kinship with *B. welchii*.

There are, however, a number of anaerobic organisms which have been described from time to time by various workers and which seem to present certain features in common with those that characterise *B. welchii*. These may be divided into two groups, namely:

Group I.—The individual members of this group are non-motile, give rise to stormy fermentation in milk, and do not form spores in this medium. The group comprises.

1. *B. aerogenes capsulatus* of Welch and Nuttall (1892 ⁴⁰), Synonym *B. welchii*.
2. The bacillus of acute articular rheumatism of Achalmé (1891 ¹, 1897 ²).
3. *B. enteritidis sporogenes* of Klein (1895 ¹⁸, 1901 ¹⁹).
4. *B. perfringens* of Veillon and Zuber (1898 ³⁴).
5. *B. saccharobutyricus immobilis* of Schattenfroh and Grassberger (1900 ²⁷).
6. *B. phlegmonis emphysematosæ* of Fraenkel (1902 ⁹).

Group II.—The members of this group are characterised by motility, by stormy fermentation in milk, and by the development of spores in milk. It includes:

1. *B. amylobacter* of Gruber (1887 ¹¹).
2. *B. saccharobutyricus* of Von Klecki (1896 ¹⁷).
3. *Granulobacter butyricus* of Beijerinck (1896 ⁴).
4. A motile butyric acid bacillus described by Grassberger and Schattenfroh (1902 ²⁸).
5. *B. amylobacter* of Bredemann (1909 ⁶).

A long series of observations on pure strains of *B. welchii* taken

from wounds show these to be as constant and fixed in their reactions and behaviour in culture media as any well-established bacterial species, and for this reason we believe that the various organisms which come into Group I. are identical with *B. welchii*, such differences as appear in the published description of individual members being due to impure cultures. Nor have we so far succeeded in isolating from wounds any organism which presents the features that are common to individuals of Group II.

B. *The Morphology and Staining of B. welchii.*

In exudates, in tissues, and in young cultures, *B. welchii* is very strongly Gram-positive. The Gram-negative individuals which are to be found only in old cultures vary much in their frequency with the individual strain and with the medium used for growth. The Gram method which gives the best results for this as for the other anaerobes is the modification of Claudius described in the Appendix (p. 380).

Suitably stained preparations made from exudate and from infected tissues show the bacillus to be surrounded by a well-defined capsule, but it is not always easy to demonstrate this. Smears made from an albuminous medium may give an appearance of capsules due to shrinkage of the coagulated film away from the bacilli.

The bacillus is a straight stout rod which presents a certain resemblance to anthrax, except that it shows but little tendency as a rule to chain formation. It has slightly rounded ends, but in the short chains occasionally met with contiguous bacilli may present square-cut extremities from mutual pressure.

The average length of moderate-sized bacilli is $4\ \mu$ to $6\ \mu$, though forms two or three times as long may be frequently met with. There is as a rule a striking uniformity in size of individuals in all cases where the organism has grown in the presence of a fermentable carbohydrate. Thus preparations made from infected muscle, or from heart blood or liver after death, show bacilli which are very regular and equal in size. The same uniformity, too, is found in films made from media which have been vigorously fermented, although in this case the bacillus tends to be shorter and thicker than it is in the tissues. If, on the other hand, the organism be grown in a sugar-free medium, it may exhibit much variation in size, and it is just in old cultures of this nature that filament formation occurs. Certain figures illustrate these points. The preparation shown in Plate XXXII. Fig. 36 was taken from an 18-hour growth on agar smeared with alkaline albumin, and it already exhibits at this early stage a lack of uniformity in the size of individuals. The same strain, grown for three days on meat, which contains glucose, gave rise to short, thick, almost coccoid forms (Plate XXXII. Fig. 37), and these, when transferred to digested

casein, gave rise to occasional filaments after eight to ten days' incubation.

It becomes difficult, therefore, to describe an organism which is so pleomorphic. The following statements may be taken as a broad summary of the evidence in regard to morphological changes in *B. welchii*.

1. When the organism grows rapidly, as in the presence of a fermentable carbohydrate, the resulting individuals are very even in size.
2. In sugar-free protein-containing media, where growth is much slower, there is a tendency to filament formation.
3. The bacillus in rapidly growing cultures is as a rule shorter and thicker than it is in the tissues.

B. welchii occurs singly or in pairs. The diplo-bacillary forms, arranged end to end in a straight line or at an obtuse angle, we regard as being very characteristic of the organism. Short chains are at times met with in infected exudates, and less commonly in cultures. Plate XXXII. Fig. 38 represents a strepto-bacillary form as it occurred in a hæmothorax fluid, together with the appearance it presented in culture.

To involution forms of *B. welchii* we have paid little attention. Spindle-shaped presporing forms may be found at times in pus and more regularly in those media in which spore formation occurs. Club-shaped forms and large bloated coccoid forms are occasionally seen in cultures. Plate XXXIII. Fig. 39 depicts a curiously irregular form of *B. welchii*. The "unhatched tadpole" shapes here shown are probably identical with those mentioned by Simonds (1915²⁹). The organism was grown under bad conditions as regards anaerobiosis.

The spores of *B. welchii* are large oval structures which are considerably broader than the bacillus (Plate XXXIII. Fig. 40). They may develop in the median or subterminal position. Spore formation occurs only under restricted conditions, and would seem to be completely inhibited by the presence of acid either added to the medium or formed by the organism itself as the result of fermentation. Sporulation never occurs, therefore, in a medium which contains a fermentable carbohydrate. It takes place in alkaline media which are sugar-free and rich in protein, such as casein digest, alkaline egg albumin, or inspissated serum. The tendency to form spores varies much with individual strains. In some there is rich sporulation after twenty-four hours' growth on solid serum, whereas in others spores may be difficult to detect microscopically after a week's incubation. Where they cannot be found in films, their presence may be demonstrated by reinoculating portions of culture after heating to 80° C. for twenty minutes.

Sporing *B. welchii* is to be found with regularity in fæces and in the majority of open suppurating wounds. On the other hand, spores are seldom found in infected muscle, liver, kidney, spleen, subcutaneous

œdema fluid, cerebro-spinal exudate, or hæmothorax fluid. In those relatively few instances where sporing *B. welchii*, as demonstrated by the heat test, have been found to exist in juices and fluids taken from the sources above mentioned, the organism has been grossly mixed with other infecting microbes.

C. *The Cultural Reactions of B. welchii.*

1. *The structure of superficial and deep colonies.*—The appearance of the surface colonies of *B. welchii* depends on such factors as the character of the medium, the humidity of the atmosphere in which the organism is grown, the thickness of the seeding, and the degree of anaerobiosis maintained. The ordinary laboratory agar with a reaction of +10 to +12 to phenol-phthalein, gives uncertain growths, nor does 1 per cent. glucose agar give greater constancy in result, for in each case the harvest of colonies is not proportionate to the number of implanted microbes. If, however, either of these media be smeared with alkaline egg albumin (see Appendix) at the time of seeding, and if the implantation be made from a young vigorous culture a few hours old, then the yield in colonies is enormously increased and a very vigorous growth is obtained after eighteen to twenty-four hours' incubation.

Young rapidly growing colonies of *B. welchii* are semi-transparent and of an opalescent tint. The main part of the colony is made up of a central slightly raised portion, which is granular and ground-glass-like under magnification, and which is surrounded by a narrow refractile rim (Plate XXVIII. Fig. 22). The whole colony is very moist and has a practically circular outline with smooth even margins. Adjacent colonies tend to coalesce, and if the organism be thickly seeded a uniform surface sheet of growth results which is not unlike a vigorously growing *B. coli*. Isolated colonies, twenty-four hours old, may have a diameter of 2 mm. and may attain a diameter of 4 mm. in forty-eight hours. Plate XXVIII. Fig. 23 represents old colonies which have been kept in the incubator for one month. In these the central boss has become more opaque; the spreading margin has become wider and slightly granular. The crenated edge of the colonies depicted is a distortion effect due to drying of the agar in air after the plate was dismantled.

With repeated subcultures the opalescent coli-like colony gives place to one which becomes more and more opaque, so that it ultimately bears some resemblance to a dense white staphylococcus colony (Plate XXVIII. Fig. 24). It is more heaped up and of greater thickness than its predecessor, and may present irregular margins. There is also little tendency to coalesce with this opaque type of colony, so that contiguous colonies distort each other by mutual pressure. Individual strains of *B. welchii* show much variation in

their readiness to give this particular type of growth. Some react in this respect immediately with the first plating out after being taken from the body, while others react only after a long sojourn in artificial media.

The colonies so far described, the opalescent and the opaque types, can be picked off the agar surface just as easily as can *B. coli* or a staphylococcus, but there is another variety of *B. welchii* (Plate XXVIII. Fig. 26) which sticks with great tenacity to the medium. This variety presents a colony which shows a very thick opaque centre with a granular and slightly crenated margin. The picking off of these colonies is often attended with some difficulty, for they are very viscid in consistency and may be pulled out with the platinum loop into long elastic filaments, or they may require actual digging out with the portion of agar to which they adhere so firmly. These sticky colonies show very little tendency to coalesce.

A further type of surface colony is the surface variety shown in Plate XXVIII. Fig. 25. This results from the growth of *B. welchii* on a medium consisting of 10 per cent. gelatin and 2 per cent. agar in equal parts. This mixture forms a very transparent medium which does not liquefy at blood temperature, and which, when smeared with alkaline egg, gives very good growths of all strains of *B. welchii*. The speckling is due to fine tangled dendritic processes which the organism sends down as roots into the medium. These roots are densely white by reflected light and appear black with transmitted light. All the pure strains of *B. welchii* we have worked with show in varying degree this formation of roots on gelatin agar, but none have shown it on plain agar. The production of rooted colonies on plain agar is, however, a characteristic of both *B. sporogenes* and of *B. tertius*. The presence of gelatin in the medium would appear to encourage development of this character in the case of *B. welchii*, although in none of our strains even after prolonged incubation has there been found any evidence of actual liquefaction.

Deep colonies in sugar-free agar or agar-gelatin are usually lenticular, but these are not characteristic, for a variety of anaerobes give colonies of similar build. The presence of a fermentable sugar in the medium results in its disruption and dislocation by the formation of gas.

In gelatin at room temperature there is either no growth or but very slow development. In liquefied gelatin at blood heat there is moderately good growth, but in neither case, either at room temperature or at blood heat, have we found the gelatin to be digested.

On inspissated serum *B. welchii* gives either discrete round colonies or a continuous surface film of growth. Certain strains tend to "bite" into the medium, but there is no real digestion.

2. *Broth*.—Growth in sugar-free broth may be scanty and of slow development. The presence of even a trace of fermentable carbo-

hydrate accelerates growth, so that a diffuse haziness of increasing density develops in a few hours. There is at the same time a more or less vigorous evolution of gas. This gas may show itself as a chain of bubbles at the junction of the broth and the superimposed liquid paraffin, or it may appear as a stream of rising bubbles when the culture tube is vigorously tapped on the bench, or again its formation may be detected by previously introducing a small Durham tube. It results from the decomposition of the carbohydrate present and is accompanied by the production of various organic acids. It is these latter that set a limit to the growth of the organisms, for when they reach a certain titre the anaerobe ceases to reproduce itself, the evolution of gas comes to an end and the growth begins to sediment out. As a rule this precipitation begins towards the end of the second day and is complete inside a week, the broth becoming clear and transparent again.

The addition of chalk or of magnesium carbonate to a broth medium affords a means of neutralising the organic acids which have been formed. The growth in this case is much more profuse and the evolution of gas at certain stages may be so brisk as to give the appearance of a medium that is boiling.

In a sugar-free medium, such as casein-digest, there is a slight formation of gas from the decomposition of protein material, but it is very small in amount as compared with that formed from carbohydrate. In this medium, too, spores are found, though not regularly, whereas spore formation never occurs in the presence of a sugar which is attacked by *B. welchii*.

Pieces of hard-boiled white of egg which have been added to broth show no sign of digestion even after many weeks in the incubator. This is in striking contrast to the rapid digestion given by *B. sporogenes* in similar circumstances.

The sticky variety of *B. welchii*, the surface colonies of which have been already described, gives an appearance in broth cultures which merits special description. Certain strains of the organism, which at first resemble the ordinary type in that they present all the usual cultural features, develop, after a series of subcultures through liquid media, a tendency to give sticky growths. Six out of thirty pure strains of *B. welchii* have behaved in this fashion, and have shown in liquid subcultures the development of a thick stringy mucus-like substance. Gas bubbles formed in such a culture travel to the surface as slowly as if passing through oil or glycerin, and the culture may become so viscid that it cannot be sucked up into a pipette. After about a fortnight in broth these sticky cultures precipitate out as a cake at the bottom of the tube and leave the medium clear.

The strains which develop this change form spores very readily on inspissated serum, but their power to ferment sugars is in no way altered from the normal type.

3. *Milk*.—The milk used throughout these investigations has been collected into a sterile flask at a farm with as little risk of contamination as possible, brought straight to the laboratory, tubed out under a $\frac{1}{4}$ -inch layer of liquid paraffin and sterilised at 100° C. for one and a half hours. The cream gathers as a thick layer on the surface of the milk and acts as an extra air-lock. Tubes of milk so prepared remain in good anaerobic condition for ten to fourteen days and can be reheated after this period.

The fermentation by *B. welchii* of the lactose contained in milk starts within a few hours of inoculation and proceeds very rapidly. Gas formation and clotting go hand in hand. The clot, at first soft and diffuse, is soon channelled by bubbles of gas passing out towards the surface, and ultimately becomes broken up and contracted into tough irregular masses. These masses in their turn are exploded with disruptive violence by the pressure of the gas which develops in their interior. The end result is such as is shown in Plate XXVI. Fig. 1. Torn and ragged masses of clot lie scattered throughout the medium and adherent to the walls of the tube, their caves and hollows bathed in clear limpid whey, and the thick cream layer is dislocated by portions of clot which have been expelled through it upwards on to the sides of the tube above the paraffin layer. The whole picture is that of a stormy fermentation, which has been suddenly arrested and which has become fixed by tough clot at its period of maximal intensity.

This milk reaction is absolutely diagnostic of *B. welchii*. So far we have isolated from wounds no single organism which gives this reaction and which does not exhibit all the other features of *B. welchii*. The important features about the reaction are—(1) The rapidity with which it develops—it ought to be complete in twelve to fifteen hours; and (2) the stormy character of the fermentation, so that the cream layer is broken up and portions of clot are forced up through it on to the sides of the tube.

In those cases where the reaction is slow of development and does not become marked until the second day, the retardation is probably due to insufficiently anaerobic conditions, and the fermentation can be accelerated by planting into freshly prepared milk.

There is no spore formation in milk. The whole medium becomes very acid and has an odour of butyric acid. The organism soon dies off in the whey portion of the milk, but may live for ten to twelve days in the interior of the masses of clot.

There is never any naked-eye sign of the digestion of the casein clot.

4. *Alkaline minced meat medium*.—The medium used is that described by Miss Muriel Robertson (1916²⁵). The glycogen of muscle is after death converted into glucose and isomaltose, and it is to these that the medium owes its carbohydrate content.

B. welchii grows in this so rapidly that gas formation is well marked after two to three hours' incubation. When fermentation is in full development the whole medium has a hazy appearance, and the liquid part above the beef becomes opalescent and turbid. If the pieces of meat are tightly packed together, the gas which forms in the crevices may be sufficient to lift the whole mass up the tube so that it projects through the paraffin layer. The reaction comes to a standstill after two or three days, and the organisms are precipitated down from the liquid part of the medium, which becomes a bright canary yellow or almost orange colour, while the beef underneath is of a brick-red tinge (Plate XXVI. Fig. 2). This appearance is a characteristic cultural feature of *B. welchii*. There is never any blackening of the meat or putrefactive odour such as one finds with *B. sporogenes*.

Certain strains of *B. welchii*, if incubated in meat for ten days and then left in the ice-chest for a further ten days, show at the end of this time small white pearly masses of acicular crystals which are soluble in ammonia and which we take to be tyrosin. This precipitation of tyrosin may be hastened by actual freezing followed by thawing. It is very slight in amount compared with that produced by *B. histolyticus* under the same conditions, and is the only naked-eye evidence of digestion given by *B. welchii* that we have been able to discover.

The organism as a rule spores in meat after five days, but this is not constant.

5. *Alkaline egg albumin*.—The inoculation of *B. welchii* into this medium gives rise to the development in three or four days' time of a fine delicate network of clot. This may be diffuse or it may start at the bottom of the tube and spread upwards towards the surface so that after about a week the whole medium becomes opaque and white. The clot is always very soft and friable and does not contract or precipitate out (Plate XXVI. Fig. 3). The addition of a fermentable sugar, even in traces, to this medium results in a minor stormy fermentation with the formation of tough spongy clot.

B. welchii spores rapidly in this alkaline egg, and strains may be kept in it without subculturing for six months.

B. sporogenes.

A. The Identity and Relationship of B. sporogenes.

We have decided to call the second most frequent anaerobe found in wounds *B. sporogenes*. It is a motile organism, vigorously proteolytic, and gives rise to spores in all the media in which it develops.

The exact position of *B. sporogenes* is a matter of some importance, for with it there is wrapt up the question of the identity of the bacillus of so-called malignant oedema. *B. sporogenes* was described by Metchnikoff (1908²¹). He found it in cases of diarrhoea and also

in normal fæces, and was of opinion that there existed two separate types of the bacillus, which he called A and B respectively. In variety A, which was obtained from healthy stools, the bacillus presents itself in filaments and chains. It is relatively thin and gives subterminal spores. Variety B, isolated from the stools in cases of chronic colitis, is a very much thicker bacillus and produces median spores. This form grown in meat produces a black pigment which can be found actually inside the bacillus.

Both forms take the Gram stain, are feebly motile though provided with numerous flagella, and readily form spores in all media. Further, they give rise to woolly colonies in glucose agar, give complete peptonisation of milk, and digest gelatin and solidified serum very rapidly. Metchnikoff thought his *B. sporogenes* to be closely related to Klein's *B. enteritidis sporogenes* (1895¹⁸) and to Bienstock's *Putrificus coli* (1906⁵), and placed all three organisms together in a group of putrefactive bacilli.

Klein's organism was isolated by him from fæces in an outbreak of diarrhoea. A diagnostic feature of the organism was the reaction it gave in milk, a reaction identical with that produced by *B. welchii*. In each case there occurs a stormy fermentation with separation into a whey, having the odour of butyric acid and a tough curd which is torn into irregular masses by the gas that is formed. But Klein's bacillus presented these distinguishing features from *B. welchii*, that it was slightly motile and gave rapid liquefaction of solidified serum and of gelatin. Its description then as given by Klein showed it to be an organism which was both vigorously saccharolytic and proteolytic. The subsequent history of this bacillus is of interest, for after it had held the field for a number of years as a separate bacteriological entity, it was discovered to be really a mixture. The milk reaction was due to a non-motile bacillus identical with *B. welchii*, while the proteolytic activities shown in the culture were due to a motile organism which Klein (1901-2¹⁹) himself called *B. cadaveris sporogenes*. The latter according to the literature agrees in all its culture reactions with *B. sporogenes*.

Bienstock's *B. putrificus* also agrees with *B. sporogenes* in its cultural features, but it is described as being a long slender bacillus with round terminal spores and therefore as bearing a resemblance to *B. tetani*.

At a much earlier date, 1881, long before the above-mentioned organisms were described, Koch (1881²⁰) and Gaffky (1881¹⁰) worked with a bacillus which they called *B. oedematis maligni*. They gave its morphological features and its reactions in the animal body, but never succeeded in growing it, so that we have no knowledge of its cultural features. Many years afterwards Jensen (1903¹⁶) studied a similar bacillus, and more recently Von Hibler (1908¹⁵) has given a more complete account of the same organism. Both of these observers retained the name *B. oedematis maligni* of Koch.

There is still much confusion as to the kinship of this organism with Pasteur's *Vibrion septique*, and the two have been taken to be identical by numerous writers. Such strains, however, of *Vibrion septique* as we have had the opportunity of investigating are saccharolytic, and in this respect are quite distinct from the proteolytic organism so frequently met with in wounds. Pasteur's organism is probably closely related to the bacillus of Rauschbrand, and this would

seem to be confirmed by the serological findings of Nicolle, Cesari, and Raphael (1915²³).

One is reduced then to a choice between two names for the motile proteolytic organism so frequently isolated from wounds. We may call it *B. oedematis maligni* of Koch after German writers, or we may name it *B. sporogenes* of Metchnikoff, and thus accept the description of French writers who have never recognised the name *B. oedematis maligni*.

The difficulty of reproducing a clinical picture of so-called malignant oedema after inoculation of pure cultures of this proteolytic organism in animals, and the ease with which such a result follows the inoculation of proteolytic organisms mixed with such an organism as *B. welchii* (see Robertson) make one doubtful of the existence of malignant oedema as a definite bacteriological entity.

It is preferable for this reason, we believe, to discard the name of malignant oedema, and to retain the name of *B. sporogenes*, for an organism which gives the cultural reactions hereinafter detailed. It is not unlikely that one is dealing with a whole group of bacilli rather than with an individual; but we have not so far found that the individual members of the group, if they exist, give distinctive cultural features, although it may ultimately be possible to differentiate them by serum reactions.

B. *The Morphology and Staining of B. sporogenes.*

In contradistinction to *B. welchii* this organism is actively motile, but individual strains vary much in this respect. Motility is a marked feature in some strains, where even young sporing individuals may be seen to move actively across the field, whereas in other strains only an occasional motile bacillus can be found. In certain of these latter long filamentous forms may at times be observed to hurry across a field of motionless organisms with the rapid sinuous snake-like movement described in the case of Pasteur's *Vibrio septique*.

In the tissues the organism, both in its vegetative phase and in the sporing condition, is as a rule strongly Gram-positive. It may, however, stain irregularly, the chromatin being distributed through the individual bacillus in question in small discrete masses, so that the organism presents a granular or irregularly beaded appearance. Gram-negative forms are relatively common in old cultures.

The bacillus is almost the same length as *B. welchii*, and may have the same thick appearance in the tissues, but in cultures it is as a rule considerably narrower than *B. welchii*. Sinuous filaments or segmented chains of bacilli are found in the tissues and also in cultures in liquid media. These long forms are much more frequent with *B. sporogenes* than with *B. welchii*.

The characteristic feature, however, of the organism is its readiness

to form spores, both in the animal body and in cultures. The spores (Plate XXXIII. Fig. 41) may be central, subterminal, or terminal. While still attached to the bacilli they are oval, but tend to become more spherical when free. Strains which tend to grow as short bacilli would seem to run to spore formation much more readily than those which give rise to filamentous and streptobacillary forms.

The ease and rapidity with which this organism forms spores in artificial culture is in striking contrast to the behaviour of *B. welchii* under similar conditions.

C. *The Cultural Reactions of B. sporogenes.*

1. *The structure of superficial and deep colonies.*—All organisms of the *B. sporogenes* type present woolly colonies when grown on the surface or in the depths of solid media. The very young surface growth (Plate XXIX. Fig. 27) gives the agar plate no more than a dull ground-glass-like appearance. This when examined under moderate magnification discloses a multitude of small transparent colonies which are contorted into a variety of shapes, and which show commencing development of the radiating processes that give the fully developed colonies their typical appearance. After two or three days' incubation, these small transparent colonies become visible to the naked eye. They show an opaque granular centre, from the edges of which springs a tangled network of gnarled branches (Plate XXX. Fig. 31). It is this arborescence that gives the older colony its woolly character. The development of these filaments seems to be determined partly by the motility of the particular strain of organism and partly by the amount of moisture on the surface of the inoculated plate.

With dry plates and an organism of feeble motility, the colony has a dense granular centre and a relatively narrow margin of filaments, whereas a motile organism on a moist surface gives rise to colonies with a relatively smaller centre and a wider zone of peripheral branching (Plate XXIX. Fig. 28).

Deep colonies develop more slowly than do surface growths; they are much more opaque as a rule, and the marginal network of filaments is more limited in its extent.

2. *Broth.*—There is much variation in the luxuriance of growth in broth cultures. Some strains give a diffuse cloud very rapidly in ordinary broth, while with others a slight bottom turbidity may be all that is visible after forty-eight hours. The addition to the broth of glucose, which gives such vigorous development in the case of *B. welchii*, does not seem to accelerate the growth of these more slowly growing proteolytic strains. They will, however, develop well in broth which contains even small amounts of alkaline egg albumin.

Pieces of hard-boiled white of egg in broth are completely digested in ten to fourteen days. Another convenient method of showing the

proteolytic activities of this *sporogenes* type of organism is to add to the broth pieces of cooked crab or lobster muscle. The coarse muscle bundles swell up, lose their distinctness, then crumple up and dissolve away to form a granular detritus. The growth of all strains in broth is characterised by a strong putrefactive odour. This is never present in the case of *B. welchii*.

3. *Milk*.—Cultures in anaerobic milk begin to show a separation of whey during the second day of incubation. The casein is first precipitated in fine shreds and then finally digested without any previous coagulation *en masse* or production of gas. After five to ten days the culture presents the appearance depicted in Plate XXVI. Fig. 4. The cream layer is undisturbed, there is a small amount of débris at the bottom of the tube, and the rest of the medium consists of yellow turbid whey. This peptonisation is in marked contrast to the stormy fermentation produced in milk by *B. welchii*.

4. *Alkaline meat*.—This is a particularly favourable medium for the growth of *B. sporogenes*. In twenty-four to thirty-six hours there is evolution of gas, but it is always in much smaller amounts than in the case of *B. welchii*, the liquid part of the medium becomes turbid and the particles of meat swell up, look sodden, and have a dirty dull purple tint. Digestion proceeds rapidly so that after five to ten days the meat is much diminished in volume (Plate XXVI. Fig. 5), it is blackened and the rest of the culture consists of a foul-smelling turbid liquid.

This blackening of meat is a very important reaction. We have not been able, so far, to determine the nature of the pigment that is formed. It may be a tyrosin derivative, such as that which results from the interaction of tyrosin with tyrosinase; or it may be a black humin substance resulting from the condensation of an amino-body with a sugar.

In view, however, of the production in these cultures of sulphuretted hydrogen, the presence of which can be readily shown by the blackening of filter paper moistened with lead acetate solution, we have thought it more likely that the pigment is really a sulphide of iron, the iron being that which is set free in the breaking down of hæmoglobin. We have as yet, however, no absolute proof that it is a sulphide of iron.

The pigment is deposited in the particles of meat, and also on the sides of the test tube, where it not infrequently forms a black ring just under the paraffin layer.

The meat may be blackened diffusely, as in Plate XXVI. Fig. 5, a culture of a vigorously proteolytic strain, or only its upper layers may be thus pigmented, as in Plate XXVI. Fig. 6, a less actively proteolytic strain.

Another feature of meat cultures of *B. sporogenes*, especially if these have been left in the cold for several days, after incubation, is

the separation of balls of a crystalline substance which we assume to be tyrosin, but this is never so marked as in the case of Weinberg's *B. histolyticus*.

5. *Alkaline egg albumin*.—The first result of growth in this medium is very similar to that produced by *B. welchii*. The medium becomes more and more opaque, the process usually spreading from the bottom of the tube upwards towards the surface, so that the albumin is ultimately completely clotted. Later the clotted cylinder of albumin becomes detached from the sides of the tubes, and as digestion proceeds it shrinks and falls to the bottom of the tube (Plate XXVI. Fig. 7) and is slowly dissolved.

6. *Inspissated serum*.—This is completely liquefied in a few days.

B. tertius.

A. The Identity of B. tertius.

The organism has been described by Fleming (1915⁸) under the designation of *Bacillus Y*, and also by Miss Robertson, who regards it as being identical with bacillus IX. in Von Hibler's series (1908¹⁵), and also with an organism found by Rodella (1902²⁶) in the stools of infants. In point of frequency it is the third anaerobe found in wounds at the Base Hospital in France, and we have called it *B. tertius* for this reason.

It is an end-sporing bacillus which is strongly saccharolytic, and which must therefore be responsible for gas formation in the tissues, but it is said to be non-pathogenic when inoculated in pure culture into animals. We think it likely that here, just as in the case of *B. sporogenes*, we are dealing with a group of organisms rather than with one individual species.

B. Morphology and Staining of B. tertius.

It is a very thin bacillus (Plate XXXIII. Fig. 42) which is often slightly curved and which may frequently have a granular or beaded appearance due to irregular staining. In the tissues and in young cultures it is Gram-positive, but rapidly loses this property after a few days' growth. It spores rapidly under all conditions, giving rise to a large oval terminal spore which may be actually longer than the bacillus to which it is attached. By spore-staining methods the free spores are found either to be solidly stained or to decolorise irregularly as if the chromatinic material were unevenly aggregated inside the spore shell. The attached spores are of two types. The young developing spore is a small spherical or slightly oval terminal structure which retains the stain well, whereas the large fully developed spore is often easily decolorised. The latter may be no more than a

spore shell or husk from which the attached bacillus has already emerged.

The organism is non-motile or only feebly motile.

C. *The Cultural Reactions for B. tertius.*

1. *The structure of superficial colonies.*—Surface cultures of *B. tertius* give rise to good growths after two to four days, the colonies being fully developed after five to six days' incubation. Two types of colony are met with. Type A (Plate XXIX. Fig. 29) is a very fine transparent colony which may be overlooked on naked-eye examination. With magnification it is found to have a clear centre and a slightly irregular margin which is finely granular. Type B (Plate XXX. Fig. 32) is a larger colony, slightly less transparent, and having a border which may be markedly crenated or which may send out small irregular offshoots. This particular form of colony may have an opaquely granular centre from the development of fine roots sent down into the depths of the agar.

In the case of the majority of strains which we have isolated in pure culture, the early two days' old colony is of type A, and this after two or three more days' incubation gives rise to a colony of the B type. In certain strains, however, there is little or no tendency for the A type of colony to grow into the B type. Those organisms which go no further than the A type show no motility, whereas those which give the larger crenate and rooted colonies are slightly motile.

2. *Broth.*—In plain broth there is a good growth in twenty-four hours, and the culture shows a uniform turbidity which settles out as a deposit after four or five days. Glucose broth gives vigorous fermentation with the production of much acid and gas. Pieces of cooked egg albumin added to the broth are not attacked even after weeks of incubation.

3. *Milk.*—Freshly isolated strains of *B. tertius* produce no change in milk in twenty-four hours, other than the formation of a small amount of gas. In forty-eight hours a soft friable coagulum is formed, and this (in the next few days) may be slightly fractured or tunnelled by gas (Plate XXVI. Fig. 8). With fresh vigorous strains the clot may contract so as to express a moderate amount of clear whey. Strains which have been isolated from the body for some time give a slow milk reaction which may take a week to develop.

4. *Alkaline meat.*—The organism grows well in meat, producing acid and gas in twenty-four hours, but never quite so vigorously as does *B. welchii*. At the end of three to six days, the fermentation subsides, the fluid part of the culture clears, while the meat takes on a bright rose tint (Plate XXVI. Fig. 9). In older cultures left in the incubator for two to three weeks the upper layers of the meat become decolorised, probably as the result of an oxidation process.

The intensity of the colour produced in meat by a saccharolytic organism seems to depend upon the amount of acid formed and therefore on the vigour of the fermentation. In the case of *B. welchii* the reddening is deeper and lasts longer than with the other members of the series. The pigment is, we believe, some acid derivative of hæmoglobin. The larger the amount of acid formed, the more fixed and stable is this pigment, and the less liable it is to be decolorised by oxidation. In the case of *B. oedematiens*, described later, which ferments glucose much less vigorously than does either *B. welchii* or *B. tertius*, the meat takes on a pink colour which is quickly destroyed by an oxidation process which starts at the surface and passes right down to the bottom of the tube.

5. *Alkaline egg*.—The medium goes opaque in five to ten days, and in more recent strains the coagulum may shrink from the sides of the tube and express a clear watery fluid (Plate XXVI. Fig. 10). In most cases, however, there is no more than the development of an opacity as in the case of *B. welchii*, and even this may be limited to the bottom of the tube. There is no sign of digestion.

6. *Inspissated serum*.—On solid blood serum there is good growth of isolated or of confluent colonies, and certain strains show a tendency to bite into the medium, but there is never any liquefaction.

B. fallax.

A. The Identity of B. fallax.

The organism was first isolated and described by Weinberg (1915³⁹). He has named it *fallax* because of the difficulty he experienced in differentiating it from *B. welchii*. It is very vigorously saccharolytic and shows very little proteolytic activity under artificial conditions.

B. Morphology and Staining.

Compared with *B. welchii* it is a relatively short thin bacillus having a length of $2\ \mu$ to $6\ \mu$ and a width of $0.5\ \mu$ (Plate XXXIV. Fig. 43). It has rounded ends, and in cultures may be slightly curved. The organisms lie scattered irregularly over the field, there being no particular grouping either in twos or in chains. It is feebly motile in young cultures, and is said to be actively motile in the body. Weinberg describes the presence of a large number of very long cilia, and states also that the organism may be capsulated in the tissues. It is Gram-positive.

Spore formation occurs occasionally in alkaline meat, and with greater regularity on inspissated serum, the spores being small oval structures which are usually subterminal.

C. Cultural Reactions.

1. *The structure of surface colonies.*—The surface growth of *B. fallax* on ordinary agar smeared with alkaline egg-albumin is in the first twenty-four hours much like that of a slow-growing *B. welchii*, except that the colonies are more transparent. On the second and third day the colonies (Plate XXIX. Fig. 30), still very transparent and having a diameter of 1 to 2 mm., are found to have more irregular margins than those of *B. welchii*. The edges of the colonies may be quite crenated, and in addition certain of the larger colonies may develop a central refractile bubble of gas. Colonies can be easily picked off from the agar surface, and there is no tendency to the formation of roots.

2. *Broth.*—Broth cultures are identical with those of *B. welchii*, growth being accelerated by the presence in small amount of any fermentable carbohydrate. On the second day the organism begins to deposit out, and in three or four days the culture medium becomes quite clear again.

3. *Milk.*—In a casein digest medium containing lactose (see sugar reactions) *B. fallax* either produces no fermentation at all, or it attacks the lactose only after many days' incubation. The fermentation of the lactose in milk is also a late phenomenon, so that the milk may show gas and clot formation only after a long interval.

An active strain after several subcultures gives a milk reaction in four or five days (Plate XXVII. Fig. 12), whereas in the case of other strains the clot and gas formation may not develop for two or three weeks (Plate XXVI. Fig. 11). Milk cultures have an acid smell, but there is no odour of putrefaction and no evidence of digestion.

4. *Meat.*—The organism grows rapidly in meat with the production of much gas, and after three or four days the meat takes on the rose-red colour described for *B. tertius*. At times there is a slight blackening of the particles of meat in the upper part of the tube and the production of a faintly foetid odour, but this latter is a very variable reaction and is never constant as in the case of *B. aerofetidus*.

5. *Alkaline egg.*—Growth in this medium is very slow, and results in ten to fourteen days in an opacity which may be diffuse or which may spread gradually upwards from the bottom of the tube.

6. *Inspissated serum.*—Discrete colonies or a continuous surface film of growth are found after four or five days, with occasional spore formation after ten to twelve days. There is no liquefaction.

B. aerofetidus.

A. The Identity of *B. aerofetidus*.

The bacillus has been described by Weinberg (1916³⁸), who gave it at first the provisional name of *Bacillus D*, and later called it *B. aerofetidus*, because of the putrid odour it gave in all liquid cultures.

B. *Morphology and Staining.*

The organism is about the same length as *fallax*, that is, 2μ to 6μ , but is considerably narrower (Plate XXXIV. Fig. 44). It may be slightly curved and occasionally groups itself in short open chains. It is non-motile or very feebly motile in young cultures, but is said to be more vigorous in the animal tissues.

C. *Cultural Features.*

1. *The structure of surface colonies.*—Growths on agar are well developed after three days' incubation, the larger colonies attaining a diameter of about 3 mm. (Plate XXXI. Fig. 33). The colonies have a thick semi-opaque waxy appearance, and though at first quite round they soon develop a secondary fringe of stout lateral offshoots. A particular feature of this fringe development in the case of *B. aerofetidus* is that it may develop on only part of the periphery of the colony, so that this presents somewhat the aspect of the emblematic grenade.

2. *Broth.*—Cultures in broth grow readily and give a fairly thick uniform turbidity in twenty-four hours, which later proceeds to sediment out, leaving the broth quite clear again. Young broth cultures soon develop a foetid odour and rapidly become quite putrid.

Pieces of hard-boiled white of egg in broth become quite transparent after several weeks' incubation, without actually breaking down.

3. *Milk.*—*B. aerofetidus* gives in milk a fermentation (Plate XXVII. Fig. 13), which is almost as vigorous as that produced by *B. welchii*, and which results in the formation of spongy clot. It may at first sight be mistaken for the *welchii* reaction, but it is distinguished by several features.

- (1) The reaction develops later—in twenty-four to thirty-six hours.
- (2) It is not so intense—the clot is not forced through the cream layer up on to the sides of the tube.
- (3) The culture has a foetid odour.
- (4) The clot is slightly digested after several weeks.

4. *Meat.*—There is vigorous fermentation, which lasts for several days. The meat takes on a dull red tinge and then develops gradually a slight blackening of the upper layers. This is the only sign of proteolysis, for the meat does not diminish in volume nor have we seen tyrosin crystals precipitated out in the culture. The meat does not decolorise easily.

5. *Alkaline egg.*—A uniform opacity, the result of coagulation, develops in three to four days. The clot may later separate and become partially digested.

6. *Inspissated serum.*—There is good growth with liquefaction and the production of a very putrid odour.

B. oedematiens.

A. *The Identity of B. oedematiens.*

This organism has been isolated from wounds by Weinberg and Seguin (1915³⁷), who base their description on the investigation of six separate strains. It would seem to be highly pathogenic to laboratory animals and secretes a powerful soluble toxin. Its characteristic feature in the tissues is the production of an intense and rapidly spreading gelatinous oedema. Aschoff (1916³) and Conradi and Bieling (1916⁷) have separately described an anaerobe from wounds which resembles Weinberg's organism in this respect, but the Aschoff-Conradi bacillus would appear to have saccharolytic reactions which distinguish it from *B. oedematiens*.

We have not as yet succeeded in isolating it from wounds, and our experience with the bacillus is limited to the investigation of two strains kindly sent us by Weinberg.

B. *Morphology and Staining.*

In the tissues and in young cultures it is Gram-positive but loses this property in older growths. It is slightly motile under strict anaerobic conditions, being provided with a relatively large number of flagella. It is about the same thickness as *B. welchii*, 0.8 μ , but it is much more pleomorphic. According to Weinberg's description it appears in three different aspects in films made from cultures. Most frequently the bacillus is slightly curved into a C or S shape, the straight individuals being less common. Or it may present a preponderance of strepto-bacillary forms. Much of this variation would seem to depend on the conditions of growth. The sporing form with short chains shown in Plate XXXIV. Fig. 45A was taken from a 6-day broth culture, and this same strain grown in surface colonies on agar gave the curved and filamentous form seen in Plate XXXIV. Fig. 45B.

Oval spores, subterminal in position, are produced fairly readily in all media, but spore formation is never a marked feature as it is in the case of *B. sporogenes*.

C. *Cultural Features.*

1. *The structure of surface colonies.*—Surface growths on agar obtain full maturity in three to four days. The larger colonies (Plate XXXI. Fig. 34), 2 to 3 mm. across, present a very opaque granular centre and a relatively wide semi-transparent border which is fringed with delicate offshoots. The adult colony is distinguished from that of *B. aerofetidus* in that it is more symmetrical, and it has

not the waxy appearance of the latter, but is differentiated into an opaque centre and a transparent periphery. With *B. oedematiens* also the marginal arborescence is finer and more delicate in its structure.

2. *Broth*.—The organism grows scantily in broth. In six to eight hours there develops a slight cloud which may be so indefinite that it readily escapes notice. In twelve to twenty-four hours the bacilli fall together into clumps, and the resulting flocculi deposit out on the succeeding days of growth. There is no digestion of pieces of cooked egg-white.

3. *Milk*.—There is no change in milk for a week or ten days. Then the casein begins to precipitate out in fine grains leaving a very narrow zone of turbid whey beneath the cream layer. After several more weeks in the incubator, the small shreds of casein clot may deposit still further, but the whey never clears. The reaction after four months in the incubator is depicted in Plate XXVII. Fig. 15. It resembles an early *B. sporogenes* reaction in milk.

4. *Meat*.—Good growth in meat occurs within twenty-four hours and continues for two or three days. The evolution of gas is as a rule very slight and the meat is turned a pale red tint. It is afterwards decolorised by an oxidation process, spreading downwards from the surface of the medium (Plate XXVII. Fig. 16). This bleaching of the meat occurs more readily and with greater constancy in the case of *B. oedematiens* than with any other of the anaerobes herein dealt with.

5. *Alkaline egg*.—This is coagulated gradually after five to ten days' incubation, a soft friable clot being produced first in the lower reaches of the tube and then spreading towards the top. There is no sign of digestion.

6. *Inspissated serum*.—Good growth occurs in two to four days with biting of the medium, but there is no real digestion.

B. histolyticus.

A. The Identity of B. histolyticus.

The organism was first described by Weinberg and Seguin (1916⁸⁶). We have not so far obtained it in pure culture from wounds, and our investigations are limited to one strain provided by Weinberg. The organism is much more vigorously proteolytic in some respects than is *B. sporogenes*, and it presents differential cultural features from the latter. When inoculated intramuscularly into the thigh of a guinea-pig it produces rapid and complete digestion of all the tissues, so that in twenty-four hours, if the animal survives, only the bones of the limb are left intact.

B. Morphology and Staining.

It is a short Gram-positive bacillus, 2 to 6 μ long, and about 0.5 μ broad, and frequently grouped in pairs. It is very motile in the tissues and in young cultures, and shows many flagella with suitable staining. It develops large subterminal spores, but cultures are never very rich in spores.

C. Cultural Reactions.

1. *Surface colonies*.—On agar the organisms tend to give a very fine continuous surface film which may be easily overlooked. Isolated colonies (Plate XXXI. Fig. 35), which may be 2 to 2.5 mm. across, after three days' incubation are very transparent structures, having the same consistency throughout from centre to periphery, and having irregularly crenated margins with occasional small offshoots. Adjacent colonies readily run together.

2. *Broth*.—In twenty-four hours there is a thick uniform cloudiness in plain broth. Growth is still more luxuriant with broth containing alkaline albumin, but less so with broth containing 1 per cent. glucose. The culture proceeds to sediment out after three days and the medium may be clear again in about a week. Broth cultures are slightly foetid.

3. *Milk*.—No gas is formed. Casein is precipitated out in fine grains and is very rapidly digested, so that in two to three days it is completely dissolved. The reaction is much more vigorous than that produced by *B. sporogenes* in milk. Plate XXVII. Fig. 17 shows a 2-day-old growth in which only a little débris is left at the bottom of the tube, the rest of the medium consisting of a transparent yellow fluid surmounted by an undisturbed layer of cream.

4. *Meat*.—In twenty-four hours there is vigorous growth in meat with setting free of a slight amount of gas. The particles of meat become swollen at first, just as in the case of *B. sporogenes*, and may in the course of the next two days diminish slightly in volume. About the fourth or fifth day dense white balls of what is probably tyrosin begin to be precipitated out, and these may become so numerous as almost to obscure the meat. After several weeks the culture may appear to be "snowed up" with these crystalline masses. This appearance in meat we have not found with any other anaerobe, and it is probably characteristic of *B. histolyticus* (Plate XXVII. Fig. 18).

5. *Alkaline egg*.—In two days a fine soft diffuse coagulum is developed. Later this clot separates from the walls of the tube by contraction and is gradually digested.

6. *Inspissated serum*.—*B. histolyticus* gives on this a uniform surface film which soon produces liquefaction and a foetid odour.

III.

THE DIFFERENTIATION OF CERTAIN ANAEROBES FROM WOUNDS BY MEANS OF SUGAR REACTIONS.

The sugar reactions herein recorded have been carried out with a series of twenty-three fermentable substances.¹ In the case of the more readily obtainable substances we have used 1 per cent. solutions and have carried out fermentation reactions several times with each individual organism investigated, whereas in the case of the more expensive substances we have used 0·5 per cent. solutions and have had to be content with a limited number of experiments.

At the outset some difficulty was experienced in getting a suitable medium as basis or vehicle for the sugar reactions. The ordinary peptone solution used with aerobic intestinal bacteria is not of sufficient nutritive value to be suitable for anaerobic work.

After a number of experiments with various media we ultimately adopted a neutral pancreatic casein digest, kindly prepared for us by Captain Wolf (see Appendix). This medium is sufficiently rich in the digestion products of protein to give consistently good growths of the anaerobes, and it possesses a further advantage in that its neutrality reduces to a minimum the tendency of dissolved sugars to break down in the process of sterilisation.

The sugars added to this casein digest fluid in the proportion of 0·5 per cent. or 1 per cent., are dissolved either in the cold or by means of gentle heating. The solutions are then tubed out to a depth of about 1 inch into narrow tubes, which are $\frac{3}{8}$ inch in diameter and which contain a small Durham gas tube ground down so as to have an oblique open end. The medium in each case is covered with a $\frac{1}{4}$ inch layer of liquid paraffin, and the tubes are then sterilised in the steamer for one and a half hours. The fractional methods of sterilisation used for ordinary media are not sufficient for media covered with liquid paraffin.

As indicators of the presence of acidity a number of dyes have been tried, but none have been found to be satisfactory. They are either completely destroyed in the process of growth, or they are reduced to a colourless leuco-state under anaerobic conditions. The addition, however, of egg albumin to the media has proved to be a useful and reliable indicator of acidity, for the albumin is precipitated out in coarse flocculi in the presence of acid. It has been our custom, preparatory to inoculation, to add two or three drops of an alkaline egg-albumin solution to each tube, the series of tubes so treated being then placed in a boiling water bath for fifteen to twenty minutes to expel free air.

¹ *Note.*—For the gift of various sugars we take this opportunity of expressing our heartiest thanks to Dr. Blanchetière, Dr. Dale of the Medical Research Committee, Captain Hopwood, Professor Symmers, Lieutenant Thompson, and Captain Wolf.

Inoculation of the tubes has been carried out in a variety of ways. At the beginning we used saline emulsions of pure growths of anaerobes on agar plates, but we soon discarded this method as being tedious and as possessing no particular advantages. Later we used growths in the sugar-free casein digest medium itself as inoculation material. This medium is very favourable for the growth of the proteolytic anaerobes, but certain individuals of the saccharolytic group grow in it sparingly and so give rise to thin emulsions. Ultimately as a routine procedure we have come to use the liquid portion of vigorously growing meat cultures, and this method has given consistently good results. With repeated passages through meat most of the anaerobes we have dealt with give sufficiently vigorous growth to ensure the presence of a rich emulsion in the liquid part of the medium after twenty-four to forty-eight hours. A valid objection to this procedure lies in the fact that the meat emulsion contains a certain amount of glucose. To obviate this objection we have centrifuged the fluid part of the meat culture and used for inoculation a saline suspension of the bacillary deposit. We found, however, after numerous controls that as a matter of practice it was unnecessary to do this, for the amount of glucose inoculated with the meat emulsion is too small to interfere with the sugar reaction proper, provided that the material be inoculated in small quantities. Our routine has consisted in the addition to the sugar tubes of drop doses of a vigorously growing meat culture, by means of a Pasteur pipette thrust through the paraffin layer, and this procedure has been found to provide a convenient and satisfactory method of inoculation.

A positive fermentation reaction is indicated by the formation of gas which collects in the small Durham tube, and by the production of acid which precipitates the added albumin. When there has been doubt as to this precipitation of albumin, we have had recourse to neutral litmus solution added with a pipette after the full period of incubation, but it is rarely necessary to do this.

Where the reaction is frankly positive or frankly negative there is no hesitation in reading the result. It is with partial reactions that the difficulty in decision obtrudes itself, for most of the anaerobes we have investigated produce gas in a sugar-free medium as the result of proteolysis. The quantity of gas derived from this source is relatively small, and it appears at a late period in the history of the culture. Proteolysis need not be considered as a contributory factor in the matter of gas production when one is dealing with a clearly positive saccharolytic reaction, because, in the case of certain of the anaerobes, the acidity produced in the course of carbohydrate fermentation is of sufficient degree to inhibit proteolysis altogether. This inhibition of proteolysis is certainly true of most strains of *B. welchii*, but how far the statement holds good in regard to other members of the saccharolytic group it has been as yet impossible to

ascertain. In the positive reaction, then, one has to consider whether the formation of gas is due to the breaking down of the protein constituents of the vehicle; whether it is derived from the fermentable substance that is added to the vehicle; or, lastly, whether it results from a combination of both saccharolytic and proteolytic activities. In our search for the proper answer in regard to these three alternatives we have come to accept the time factor as being the determinant in the decision, *i.e.*, the formation of gas occurring within the first twenty-four hours of incubation we take to be evidence of carbohydrate fermentation rather than of proteolysis. For this reason all our reactions have been tabulated as final at the end of a two days' growth period (see Table II.).

Given proper conditions the *B. welchii* reactions are completed in about fifteen hours. A reference to the accompanying table will show that there are four distinct types of *B. welchii*, the differentiation depending on the fermentation of inulin and glycerin. Our findings in this respect agree with those of Simonds (1915²⁹), but they differ in the proportion in which these particular types occur. In our experience about half the strains isolated ferment both inulin and glycerin. We have attempted to separate these strains still further into an inulin fermenter and a glycerin fermenter, but without success. In only one instance did repeated plating result in the separation of an inulin fermenter from a parent strain which was positive to both inulin and glycerin. The absence of both inulin and glycerin fermentation would seem to be rare, for only two strains in our series show this behaviour, and one of these is a stock *B. welchii*, kindly sent us by Weinberg from the Pasteur Institute. It is impossible so far to say how far these inulin and glycerin reactions may vary with time. Certain of the inulin positive strains show a tendency to ferment glycerin, but the reaction when it occurs is feeble compared with that which is produced by the real glycerin fermenter. The same is true, too, of the glycerin fermenter which sometimes breaks up inulin feebly, but here again the reaction is inconstant and need not be confounded with the real inulin reaction. We are inclined to believe that the freshly isolated *B. welchii* from wounds ferments both substances, and that one or other reaction is first lost, and that both reactions then disappear with a sojourn in artificial media.

Of the other *B. welchii* reactions all are constant except that in raffinose, which seems to vary considerably. We have not found any relationship between the raffinose reaction and that produced in inulin and glycerin.

The next organism in the series, *B. fallax*, can be distinguished from *B. welchii* in that it does not give a lactose reaction in twenty-four hours. The lactose reaction develops after forty-eight hours, or several days, or it may be absent altogether. The positive salicin

reaction when it is well developed affords additional affirmative evidence of *B. fallax* as opposed to *B. welchii*, but where the salicin reaction is slight or indefinite, the only diagnostic feature of *B. fallax* is the retarded or absent lactose reaction.

The third organism on the table, *B. aerofetidus*, differs from the rest of the vigorously saccharolytic series in the absence of a saccharose reaction. This feature would appear to be constant, and therefore of diagnostic value in the differentiation of this organism from other members of the group. Another distinctive feature between *B. fallax* and *B. aerofetidus* would seem to lie in the fermentation by the former of starch, inulin, and dextrine, but we lay no stress on this in view of the varying composition of commercial brands of soluble starch and dextrine.

B. tertius can be readily differentiated from *B. welchii*, because of the positive reactions it gives with mannite and with salicin. In addition certain strains of *B. tertius* ferment amygdalin, and it is possible that the use of further glucosides, when these become available, may be of service in distinguishing the individuals of the *B. tertius* group. It is of interest to record that in five instances, where rapidly isolated strains of *B. welchii* were passed through the sugars and gave a positive salicin reaction, subsequent plating out has revealed the presence of *B. tertius* mixed with the supposedly pure *B. welchii*.

The remaining organism in the saccharolytic group, *B. oedematiens*, gives acid and gas in glucose, lævulose, and maltose, acid in xylose and also in starch.

The strongly proteolytic organisms, *B. sporogenes* and *B. histolyticus*, are very difficult to deal with in the matter of defining sugar reactions for the reasons already discussed. *B. sporogenes* gives early fermentation in glucose, lævulose, and maltose, while *B. histolyticus* gives acid in the same sugars.

The sugar reactions herein recorded are given tentatively. They are open to modification and alteration with further experience. It is hoped, however, that they may furnish a starting-point for the investigations of other workers.

IV.

THE CHANGES PRODUCED BY ANAEROBES IN THE TISSUES.

Our conception of the rôle played by anaerobes in the tissues has been much helped by the work of Wolf and Harris (1917⁴¹).

The anaerobe differs from the aerobe in that it produces in the presence of organic material changes which can be readily estimated by ordinary chemical methods. These changes are brought about by vigorous fermentations, in which proteins, carbohydrates, and fats are broken down into simpler products. In this respect the anaerobe in

its process of growth and development may be likened to a strong chemical reagent which is able to effect catabolic changes in the material with which it comes in contact. An investigation, then, of the behaviour of anaerobes in cultures is of value, not only in that it gives an indication of the changes which result from anaerobic infection of dead tissue, but also in that it enables one to understand the nature of the deleterious influence which these changes exercise on surrounding healthy tissue.

The conditions of modern warfare are such that the soiling of a wound by earth or by clothing infected with faecal organisms is unavoidable. Further, the short range bullet and the shell fragment bring about a destruction of tissue which gives just those conditions that are most favourable for anaerobic growth. These two factors in combination result in the implantation of anaerobes into a focus of dead tissue and so reproduce in the living body a condition which in certain respects is comparable to an artificial culture. In estimating the extent of the initial trauma one has to take into consideration, not only the amount of tissue in the wound which is directly destroyed by the missile, but also the amount of tissue in the vicinity and confines of the wound which is damaged through interference of its blood supply either by laceration of its vessels or by the pressure from hæmorrhage. It is this extra destruction which makes the wound produced by the modern missile so much more formidable than that produced by the long range bullet of the South African War.

I. The anaerobes in damaged tissues develop just as they do in artificial cultures, *i.e.*, given proper conditions the carbohydrate splitters are the first to develop, and the protein splitters come later. It is because of its carbohydrate content (about 1 per cent.) that damaged muscle forms a suitable nidus for the growth of members of the saccharolytic group. The glycogen which is found in living healthy muscle is converted after death into dextrose and a small fraction of isomaltose, both of which substances are vigorously fermented by such an organism as *B. welchii*. On the other hand, in wounds involving only the skin and subcutaneous tissues, where the carbohydrate content is represented only by that in the retained blood or serum (about 0.12 per cent.), the saccharolytic anaerobes have less chance of making headway, although they are to be found in these situations just as frequently as in the severer injuries involving muscle.

This early fermentation in muscle results in the formation of acid and gas. The latter consists of a mixture of carbon dioxide and of hydrogen in varying proportions. It often develops with astonishing rapidity and may appear clinically in four to six hours after the receipt of the wound. It is possible that in certain instances the appearance of crepitation in the subcutaneous tissues may be due to the development of anaerobes in this situation, but in view of the

difficulty with which anaerobic growth is established in normal blood serum, it is, we believe, much more likely that the collection of gas in this position results by escape from subjacent infected muscle which has been torn through at the time of the initial injury, or which has been subsequently ruptured by pressure from within. Taylor (1916³⁹) has already expressed the same opinion in regard to the origin of the gas found in the subcutaneous tissues. The gas may accumulate under very considerable pressure and thus add to the embarrassment of a circulation which is already much impeded.

Coincident with the production of gas is a formation and setting free of various organic acids. One of the reactive changes which result in tissues from the presence of acid consists in the absorption of water by the protoplasmic colloids, and this cellular imbibition combines with exudate in the areolar spaces to form the oedema which is often such a striking clinical feature of tissues infected with anaerobes. The oedema may spread far beyond the confines of the actual bacterial invasion, for it has been a frequent experience to find no organism either in films or in cultures of oedema fluid taken at some distance from the wound. This flooding of the acid tissues is in the nature of a defensive mechanism. It serves not only to dilute the acid but also to neutralise it, the latter process being effected by means of the so-called buffer-salts of the serum. As oedema increases, however, so the circulation becomes impeded, and in consequence the amount of absorption of acid by buffer-salts becomes more and more reduced.

A second reactive change produced by acid in the tissues consists in the appearance of autolytic enzymes, both saccharolytic and proteolytic in nature.

The sum of these changes is that, quite apart from the destruction produced by the missile in the wound itself, the early growth of the saccharolytic anaerobes initiates a spreading devitalisation process in the surrounding tissues, a process in which gas formation, oedema, and the appearance of autolytic enzymes all figure as causative factors.

II. So far, then, we have dealt with the early phase of anaerobic development in a wound—it might be termed the saccharolytic phase. But in the wound, just as in cultures, this early period is followed by a second phase which is characterised by the development of the more slowly growing proteolytic organisms. The tissues, already damaged by the activity of the saccharolytic anaerobes in the way we have described, are attacked by the digestive ferments elaborated by members of the second group, and their protein constituents are broken down through proteoses to amino-acids and finally into ammonia bodies. Now in culture media containing fermentable carbohydrate such an organism as *B. welchii* produces enough acid to retard and finally to arrest its own growth. If, however, calcium carbonate or magnesium carbonate be added to the medium, growth becomes much more luxuriant and the vitality of the organism is increased. Until

there is more evidence with regard to the degree of acid production in the tissues it is impossible to determine to what extent this inhibitory action of acid comes into play in the growth of *B. welchii* in the animal body. Any neutralisation, however, of acid will accelerate the growth of the organism, and we think it likely that the production of ammonia bodies from protein by such an organism as *B. sporogenes* serves this purpose and thereby provides a stimulus for the fresh development of *B. welchii*. Each type of anaerobe thus aids and favours the growth of the other type. *B. welchii* in attacking carbohydrate prepares the tissues for the inroad of *B. sporogenes*, and the latter by digesting proteins facilitates the further invasion of fresh tissues by *B. welchii*.

The gas formed in the proteolytic stage of wound infection contains sulphuretted hydrogen and certain volatile bodies, and it is these which give rise to the heavy penetrating fetid odour which is such a striking clinical feature of anaerobe infected wounds. The blackening which develops under artificial conditions in meat cultures, and which we take to indicate the formation of a sulphide of iron, is likewise to be found in wounds.

III. The third phase in anaerobic infection consists in the development of a toxæmia, the exact nature of which is as yet undetermined. The clinical picture produced is chiefly that of a rapidly increasing circulatory failure in which death may result so abruptly as to give the impression that it has been caused by pulmonary embolism. But we have not come across a single example of the latter condition in a series of over 600 autopsies on men dying from gunshot wounds. This state of rapid circulatory failure has none of the clinical features that are usually associated with acidosis, nor is there any clear laboratory evidence that the local production of acid in and about an infected wound may alter the general reaction of the circulating blood and so cause an acid intoxication of the individual. We are inclined, therefore, to look on the toxæmia as caused by the absorption of some degradation product of protein rather than of carbohydrate, and that it is a toxic protein derivative rather than acid which brings about the abrupt shock-like collapse of the circulation.¹

IV. The last stage of anaerobic growth is that of successful bacterial invasion of the blood stream. In the majority of cases this inroad occurs just at, or immediately preceding, the death of the individual. Anaerobes have been isolated in cultures from the circulating blood long before the present war by a number of investigators, including Gwyn (1899¹²), who grew *B. welchii* on several occasions from the blood in a case of chorea. Stokes (1916³⁰) during the war has described a series of cases in which anaerobic growth resulted in glucose-agar shake cultures by seeding relatively small

¹ See "Memorandum upon Surgical Shock and some Allied Conditions," issued by Medical Research Committee, February 27, 1917.

amounts of circulating blood. Another interesting evidence of the dissemination of anaerobes by the blood stream is afforded by the cases of so-called metastatic gas gangrene. A slight trauma of tissue, such as that resulting from pressure over the buttocks or from introduction of fluid subcutaneously, would seem to be sufficient to determine a focus of growth. Such instances have been recorded by Wallace (1916³⁵), Mullaly and McNee (1916²²), Taylor (1916³²), Hartley (1917¹³), and others. In our own experience this ante-mortem spread would seem to be comparatively rare, for we have found anaerobes only on two occasions out of forty cases. In many of these, although blood culture before death was negative, the blood taken a few hours after death showed anaerobes in very large numbers. The factors which are responsible for this post-mortem spread are at present very imperfectly understood.

We have to take this opportunity of expressing our indebtedness to the large number of friends who have helped us generously during the progress of this work. To the Sisters and Medical Officers of a certain General Hospital in France we express our sincerest thanks. We are indebted also to Sergeant A. K. Maxwell, R.A.M.C., whose facile skill in portraying the subjects put before him has rendered easy our task of description. Our laboratory attendant, Sergeant J. W. Clayden, R.A.M.C., has always helped us ungrudgingly. Finally, we are indebted to our friend Lieut.-Col. T. R. Elliott, F.R.S., F.R.C.P., R.A.M.C., whose constant advice and kind help have made the work a pleasure.

APPENDIX.

A. The Claudius modification of Gram staining.

1. Stain for one to two minutes in 1 per cent. aqueous methyl-violet (or in carbol-gentian violet).
2. Wash film in water and put on a half-saturated solution of picric acid for one minute.
3. Decolorise in chloroform till no more dye comes out.
4. Heat the film well till thoroughly dry and then counterstain if necessary with weak watery eosin.

B. Alkaline Meat Medium.

To 8 oz. of bullock's heart freed from fat and finely minced add the same amount of tap-water and cook slowly. Add normal caustic soda solution till the mixture is alkaline to litmus. Tube out the mixture, cover with paraffin and autoclave.

C. Alkaline Egg Medium.

Beat up the yolk of one egg and the whites of two, adding 6 c.c. of N/1 NaOH. Transfer this to a large flask and make up to 500 c.c. with tap-water. Heat slowly to 95° for one hour and a half. Then filter through cotton wool. Tube out under paraffin and autoclave. A modification which results in a much clearer medium consists in using two whites without any yolk, adding 4 c.c. of normal caustic soda, and making up to 330 c.c. with tap-water.

D. Casein Digest Medium.

To a 2-litre flask containing 1 litre of tap-water add 20 grms. of anhydrous sodium carbonate. Transfer the flask when boiling to a water-bath and add 200 grms. of casein, dusting this in gradually and shaking from time to time to avoid the formation of lumps. Then place the mixture in a large Winchester quart bottle, allow to cool down, and add 3 grms. pancreatin with 15 c.c. of chloroform. Incubate at 38° C. for five days, shaking vigorously from day to day to break up the crusts that have formed. Then add 3 more grms. of pancreatin and incubate for ten days longer. After fifteen days' digestion in all the mixture is treated with 400 c.c. of N/1 HCl, steamed for thirty minutes to drive off the chloroform, and then filtered. The filtrate is treated with 120 c.c. of N/1 NaOH, and its reaction is adjusted to $P_H = 7.3$, using either the hydrogen electrode or phenol-sulphone-phthalein as an indicator in a Marriott scale. The resulting stock broth is diluted with 2 parts of 0.5 per cent. NaCl in tap-water before use, the reaction being again adjusted if necessary.

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DESCRIPTION OF PLATES XXVI.-XXXIV.

PLATE XXVI.

- FIG. 1.—*B. welchii* (Strain 82). Milk culture, 24 hours old, to show stormy fermentation. Note the disappearance of the cream layer, the disruption of the casein clot, and the displacement of shreds of clot upwards through the paraffin layer.
- FIG. 2.—*B. welchii* (Strain Pasteur). Alkaline meat culture, 1 week old, and showing reddening of the meat. Contrast this with the normal meat in Plate XXVII. Fig. 20.
- FIG. 3.—*B. welchii* (Strain 82). Culture in alkaline egg, 5 days old. Note the diffuse opacity due to fine friable clot. There is no shrinkage and no sign of digestion. Contrast this with normal alkaline egg in Plate XXVII. Fig. 21.
- FIG. 4.—*B. sporogenes* (Strain Pasteur). Milk culture, 1 month old. To show undisturbed cream layer and complete digestion of the milk.

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.

Fig. 5.

FIG. 6.

FIG. 7.

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FIG. 12.

FIG. 13.

FIG. 14.

FIG. 15.

FIG. 16.

Fig. 17.

Fig. 18.

Fig. 19.

Fig. 20.

Fig. 21.

FIG. 22.

FIG. 23.

FIG. 24.

FIG. 25.

FIG. 26.

- FIG. 5.—*B. sporogenes* (Strain Pasteur). Culture in alkaline meat, 1 week old. To show digestion and blackening.
- FIG. 6.—*B. sporogenes* (Strain Harwood). Culture in alkaline meat, 1 week old. To show marked blackening of the upper layers of meat.
- FIG. 7.—*B. sporogenes* (Strain Pasteur). Culture in alkaline egg. To show cylindrical coagulum which has undergone gradual shrinkage and partial digestion.
- FIG. 8.—*B. tertius* (Strain 68). Milk culture, 6 weeks old. To show solid clot fissured by gas bubbles.
- FIG. 9.—*B. tertius* (Strain Benson). Culture in alkaline meat, 14 days old. To show pinking of meat and commencing bleaching on the surface.
- FIG. 10.—*B. tertius* (Strain Benson). Culture in alkaline egg. To show soft cylindrical clot which has shrunk and is slightly fissured.
- FIG. 11.—*B. fallax* (Strain Truocol). Milk culture, 1 month old, showing strong solid clot disrupted by bubbles, and with orange colour at the surface.

PLATE XXVII.

- FIG. 12.—*B. fallax* (Strain 92). Milk culture, 5 days old, showing separation into a turbid whey and a soft friable clot which is slightly disrupted by gas. There is no dislocation of the cream layer. Bubbles have collected on the air surface of the paraffin layer.
- FIG. 13.—*B. aerofetidus* (Weinberg). Culture in milk, 36 hours old, and showing disruption of casein clot by gas. The fermentation is not so vigorous as, and develops later than, the reaction given by *B. welchii*.
- FIG. 14.—*B. aerofetidus* (Weinberg). Growth in alkaline meat, 7 days old, showing reddening of the meat and slight blackening of the upper layers.
- FIG. 15.—*B. oedematiens* (Strain Domange). Growth in milk, after 4 months' incubation, to show slight separation of whey from subjacent casein flocculi.
- FIG. 16.—*B. oedematiens* (Strain Domange). Culture in alkaline meat, after 15 days, to show pinking of meat and extensive bleaching from surface downwards.
- FIG. 17.—*B. histolyticus* (Weinberg). Milk culture, 2 days old, showing complete digestion, with no disturbance of the cream layer.
- FIG. 18.—*B. histolyticus* (Weinberg). Culture in alkaline meat, 4 days old, to show digestion and formation of glistening white balls of tyrosin.
- FIG. 19.—Ordinary milk under paraffin.
- FIG. 20.—Alkaline meat medium under paraffin.
- FIG. 21.—Alkaline egg medium under paraffin.

PLATE XXVIII.

- FIG. 22.—*B. welchii* (Strain Vincent). Surface colonies, 24 hours old, on alkaline egg agar. They are 2 mm. in diameter, round, smooth, and slightly granular. ($\times 6$.)
- FIG. 23.—*B. welchii* (Strain 82). Old colonies on alkaline egg agar. They are 3 to 4 mm. in diameter, have an opaque raised centre, a relatively wide granular margin, and crenated border due to shrinkage of the agar. ($\times 6$.)
- FIG. 24.—*B. welchii* (Strain Vincent B). Colonies, 24 hours old, on alkaline agar. They are opaque, heaped up, and with irregular borders. ($\times 6$.)
- FIG. 25.—*B. welchii* (Strain Vincent). Colonies, 5 days old, on alkaline egg gelatin agar. They have a raised opaque centre, a relatively wide margin, and slightly irregular borders. The speckling is due to dense opaque roots sent down into the medium. ($\times 6$.)

- FIG. 26.—*B. welchii* (Strain Oost). Sticky colonies, 24 hours old, on alkaline egg agar. They have a wide opaque centre with granular margin and slightly crenated border. ($\times 6$.)

PLATE XXIX.

- FIG. 27.—*B. sporogenes* (Strain Pasteur). Slow-growing colonies, 2 days old, on alkaline egg agar. They are much distorted and show commencing branching. ($\times 12$.)
- FIG. 28.—*B. sporogenes* (Strain Temock). Colonies, 3 days old, on alkaline egg agar, showing relatively small opaque centres and a wide zone of peripheral arborescence. ($\times 6$.)
- FIG. 29.—*B. tertius* (Strain Sherman). Colonies on alkaline egg agar after 7 days. Note the pale centre, granular margin, and slightly irregular border. ($\times 8$.)
- FIG. 30.—*B. fallax* (Strain Trucole). Colonies on alkaline egg agar, 3 days old. They are thin transparent colonies with irregular borders. One contains a gas bubble in its centre. ($\times 6$.)

PLATE XXX.

- FIG. 31.—*B. sporogenes* (Strain Brown). Rapidly growing colonies, 2 days old, on alkaline egg agar. They show large opaque granular centres and rich peripheral branching. ($\times 6$.)
- FIG. 32.—*B. tertius* (Strain Mountney). Colonies on alkaline egg agar, 5 days old. To show crenated borders and opacities in the largest colony due to the formation of roots. ($\times 12$.)

PLATE XXXI.

- FIG. 33.—*B. aerofetidus* (Weinberg). Surface colonies, 3 days old, on alkaline egg agar. They are very waxy, have stout peripheral branching and show "grenade" formation. ($\times 6$.)
- FIG. 34.—*B. oedematiens* (Strain Domange). Surface colonies, 3 days old, on alkaline egg agar, showing densely opaque granular centre, and semi-translucent feathery margin. ($\times 6$.)
- FIG. 35.—*B. histolyticus* (Weinberg). Colonies on alkaline egg agar, 3 days old. They are very fine translucent colonies, 2 to 4 mm. in diameter, and have irregularly crenated borders. ($\times 6$.)

PLATE XXXII.

- FIG. 36.—*B. welchii* (Strain 82). Film preparation, stained by Gram, of 18-hour-old culture on alkaline egg agar. To show formation of short chains and irregularity in size of individuals. ($\times 1300$.)
- FIG. 37.—*B. welchii* (Strain 82). Film preparation, stained by Gram, taken from 3 days' growth in alkaline meat, and showing short coccoid forms. ($\times 1300$.)
- FIG. 38.—*B. welchii*. A=film preparation from infected hæmothorax fluid, showing strepto-bacillary forms. B=film preparation of the same organism grown on alkaline egg agar for 24 hours, and showing filamentous and chain forms. Both stained by Gram. ($\times 1300$.)

PLATE XXXIII.

- FIG. 39.—*B. welchii* (Strain Terris). Film preparation, stained by Gram, of 24-hour culture on alkaline egg agar, to show involution forms.
- FIG. 40.—*B. welchii* (Strain Jones I). Film preparation, stained by Gram, taken from 2 days' growth on inspissated human serum. To show rich spore formation. ($\times 1300$.)

FIG. 27.

FIG. 28.

FIG. 29.

FIG. 30.

FIG. 31.

FIG. 32.

FIG. 33.

FIG. 34.

FIG. 35.

FIG. 36.

FIG. 37.

A

B

FIG. 38.

FIG. 39.

FIG. 40.

FIG. 41.

FIG. 42.

FIG. 43.

FIG. 44.

FIG. 45.

FIG. 41.—*B. sporogenes* (Strain Pasteur). Film preparation, stained by Gram, taken from growth in broth after 4 days. To show richness of spore formation. ($\times 1300$.)

FIG. 42.—*B. tertius* (Strain Tingcombe). Film preparation, stained with carbol-fuchsin decolorised with weak acetic acid and counter-stained with methylene-blue, taken from 5-day-old colony on alkaline egg agar. Note the granularity in the bacilli, and the irregular staining in the free spores. ($\times 1300$.)

PLATE XXXIV.

FIG. 43.—*B. fallax* (Strain Truocol). Film preparation, stained by Gram, taken from young broth culture 2 days old. ($\times 1300$.)

FIG. 44.—*B. aerofetidus* (Weinberg). Film preparation, stained by Gram, of 3-day culture in alkaline egg broth. ($\times 1300$.)

FIG. 45.—*B. oedematiens* (Strain Domange). Film preparations, stained with carbol-gentian violet. A=strepto-bacillary and spore formation in egg broth. B=curved forms and filaments from surface colony. ($\times 1300$.)