SOME PROTEOLYTIC ANAEROBES ISOLATED FROM SEPTIC WOUNDS.

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(With 2 Text-figures.)

During four months' work with anaerobes, which were derived from wounds, a number of species have been noticed which appear to be hitherto undescribed. Two of these are proteolytic organisms and are not pathogenic to guinea-pigs when administered in large doses.

Owing to the extreme difficulty of getting pure cultures of anaerobes with ordinary technique, isolations were attempted by Miss M. Robertson and myself by means of Barber's method (*Philippine Journ. of Science*, vol. B IX. 1914, p. 307). Our results were very satisfactory. Instead of plating, isolations were made at random from the mixed cultures, some tubes receiving one bacillus and some several. The resulting cultures, if of interest, were treated in a similar manner. The type strains used for these descriptions were, to the best of my knowledge, derived from single bacilli isolated from apparently pure cultures, and they have behaved consistently in my hands.

ORGANISM I.

The first bacillus to be described has been isolated from two cultures. One case was that of a shrapnel wound of the maxilla which exuded a quantity of thick yellow pus. The sample was taken 20 days after the man was wounded and contained also *Bacillus sporogenes* (Metch.) and cocci. The second culture was taken from a pus wound located in the region of a tibia fractured by a high explosive shell. The sample was obtained 72 days after the injury occurred, and the wound had been treated with lotio rubra, permanganate, eusol, peroxide and saline. I found besides the organism in question cocci and end-sporing organisms with circular end-spores and bacilli of the morphology of *B. sporogenes* (both the ordinary variety and a smooth colonied variety) and of the morphology of *B. Hibler* IX. The organism described below was the

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dominant one in both cultures; two or three others showed, when old, forms morphologically similar to it. The type strain was derived from a single organism isolated in turn from a single bacillus culture from a glucose agar tube colony.

STAINING and MORPHOLOGY. The bacillus is usually 3—6 times as long as it is wide and it has rounded ends. It may or may not retain Gram's stain. Under uniform conditions it is consistently paler and more gram-negative than *B. sporogenes*. The spores are oval and subterminal, though a few forms are so short as to be clostridial. Sometimes in meat cultures forms with a spore at either end are common. Gram's stain is more easily washed out from the body of the bacillus than from the sporing end. The organism forms spores readily on most media. On agar plates uniform gram-negative bacilli are the rule. In old meat cultures free-swimming forms disappear, but the mass of the meat is

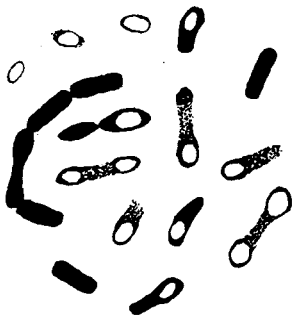

Fig. 1. Representative forms from a two day meat culture.

inhabited by enormous pale gram-negative bacilli slightly tapering at the tip and bent in wide curves. This form is the most characteristic one of the species; similar forms occur on Dorset's egg medium. On gelatine it may grow as uniform gram-negative bacilli with pointed ends, spores being absent.

MOTILITY. The organism is motile but not highly so.

CULTURAL CHARACTERS. *Meat*[1]. The growth on autoclaved meat is very characteristic. Twenty-four hours after inoculation there is usually heavy growth, the meat is pink, and some gas is present; the reaction is slightly acid. On re-incubation the meat settles more and more together with slight digestion, but it never disintegrates and never blackens. When pressed with a platinum loop it is soft and easily broken, but not

[1] Autoclaved meat medium and alkaline egg medium are described by Miss Robertson in her paper, "Notes upon Certain Anaerobes derived from Wounds," *Journ. of Pathol. and Bact.* XX. 348.

pasty and not finely granular. On long incubation it becomes bright terra cotta in colour, sometimes with a faded grey layer on top. Blackening, the general assumption of a brown or grey colour, disintegrating digestion or a refusal to digest at all are indications of impurity; on the other hand, several varieties of contaminating organisms may be present without greatly affecting the appearance of the meat. There is a putrefactive odour which is not especially characteristic.

Milk. In a hydrogen cylinder the litmus in milk is reduced if the organism grows; the milk is clotted *without* acid and slowly forms a soft irregular mass not torn by gas. Digestion of the clot begins after a few days and proceeds rather slowly; it is apparent from the yellowing of the liquid around the clot. This peculiar reaction has been consistent during long handling of the organism. The clotted milk placed on faintly blue litmus paper fails to turn it pink, and it is probable that the clotting is due to a weak rennet-like enzyme.

Alkaline egg. A fine coagulum is formed which renders the medium opaque.

Gelatine. The behaviour on gelatine, incubated at 37°, is rather irregular. Pure strains usually liquefy gelatine in two or three days although some gelatine tubes have remained unliquefied for two weeks. This irregularity is probably due to a difference in the quantity of the sowing used for the inoculation. There is a very misleading stage when a flake-like growth pervades the medium which stiffens as usual on cooling. On re-incubation for 24 hours the gelatine is liquefied.

Inspissated serum is attacked vigorously. Pitting occurs and the bases of the pits are white when many organisms are present. The surface becomes irridescent and sometimes brownish. Digestion is rapid at first but ceases before the medium is completely liquefied.

Dorset's egg medium is rendered soft and is cracked at the edges without liquefaction.

Ordinary broth and *glucose broth* are unsatisfactory media for this organism.

Action on carbohydrates. The organism was inoculated on serum litmus agar slopes containing various carbohydrates. It grew very well but failed to produce acid or gas in any case. The litmus was reduced and the mass of serum agar became somewhat pitted where the inoculation was the heaviest. The carbohydrates used were dulcite, inulin, glycerine, mannite, sucrose, glucose, maltose, laevulose and lactose. The strain used in these determinations was not the "single bacillus" strain.

Deep glucose agar tubes were excellent for the preliminary isolation of this organism. The colony in deep agar is at first round; later it takes the shape of a little worm, never growing more than 1·5 millimetres in length, and it is never woolly or radiate.

Growth on plates. This organism is easily grown on glucose agar plates. They should be dried for 20—40 minutes in the incubator before they are inoculated, and they must be exhausted and incubated immediately after inoculation. Colonies appear in 48 hours and at this period they are water-clear and very tiny. They are irregular in outline with a definite concentric ring arrangement, the middle portion being slightly granular under the microscope and the outer portion translucent. If the agar is too wet the growth will spread over the plate with fucus shaped projections.

After four days' incubation the colonies are easily visible through the substrate. They may be as much as two millimetres in diameter, are bluish grey in colour, and look to the naked eye as though they were speckled with white. Microscopically they are irregular edged and are no longer clear but are granular and wavy in texture.

Finally, these colonies are never woolly and never have fine processes; they are always minute and rarely round. They do not dig into the substrate nor are they raised or boss-like. They do not show up on the first day nor are they white or yellow.

PATHOGENICITY. Two cubic centimetres of a two days' broth culture of this organism failed to cause more than a slight swelling when injected intramuscularly into a guinea-pig.

TEMPERATURE OF GROWTH. This organism grows excellently at 37° and well also at 27° but at the latter temperature it does not start to grow as quickly. A newly inoculated meat tube shows no change at room temperature (*c.* 20°), but an old one goes on settling and reddening.

AGGLUTINATION. Bacillus I does not agglutinate with the anti-sporogenes serum of Weinberg.

Morphologically this organism would be easily confounded with *B. sporogenes* which it much resembles. The appearance of a young culture in meat medium resembles that of the acid producers, especially that of *B. perfringens*, but the organism bears so little morphological resemblance to any of those with which we are familiar that there is no danger of confusion.

ORGANISM II.

This is a typical proteolytic organism which is capable of splitting some of the single sugars as well as proteins. It has been isolated from

two cultures in this laboratory. One was taken from a very bad case of gas gangrene the material being sent from France by Capt. J. W. McNee, R.A.M.C.

The wound culture contained cocci, anaerobic bacilli of the morphology of *B. sporogenes*, *B. perfringens*, *B. Hibler* IX, an organism with circular end-spores and three apparently undescribed organisms. Organism II was first cultivated by Miss Robertson and handed over to me to describe. It was also isolated by myself from a culture from a bad gas gangrene case of Dr Brookes in the Horton War hospital. The infected leg was amputated and the patient ultimately recovered.

The other organisms found in this wound were cocci and anaerobes of the morphology of *B. perfringens*, *B. Hibler* IX, *B. sporogenes* and end-sporing organisms with circular end-spores. The strain used for this description was obtained from Capt. McNee's case and was derived from an apparently pure culture from a colony on an agar plate. Two bacilli isolated from this culture by Barber's technique gave a pure culture from which in turn a single bacillus was isolated for the type strain.

STAINING and MORPHOLOGY. The organism is more uniform in its morphology than most anaerobes. In the non-sporing state, the bacillus resembles exactly *B. perfringens*, being chunky and having very square ends. It takes Gram's stains so deeply that it usually appears black; however in a preparation from an agar slope many pale forms are seen among the dark ones. A young culture always contains many sporing individuals; after four days these may be no longer in evidence. The spores are usually central but they may be terminal. They are large in proportion to the size of the bacillus and usually markedly square at the ends. They do not bulge the sides of the bacillus to any appreciable extent and are usually clear and definite in outline. Two spores are never found in one bacillus. The size of the organism varies greatly; in a fresh meat culture the bacilli are all very large; a four days' culture has large and small forms. On broth, alkaline egg, serum and gelatine the bacilli are all much smaller than on meat. In old meat cultures and in old serum cultures long thick crooked gram-positive rods occur which are often granular.

MOTILITY. The organism is non-motile.

CULTURAL CHARACTERS. *Meat.* The reaction on this medium is very characteristic. The meat retains the colour and form that it had before inoculation but it settles somewhat in the tube. A little gas forms in the first hours and then disappears; it is usually not noticed at all. When pressed with the loop the meat in old cultures is easily disinte-

grated but is not granular or brittle. It never becomes really digested and does not spontaneously disintegrate. The liquid above the meat is yellow and perfectly clear. A putrefactive odour is noticeable.

Milk. This organism does not clot milk or render it acid. It deposits the casein in microscopic granules and then digests it very rapidly, three days' incubation being sufficient to destroy the casein. The litmus is reduced.

Alkaline egg. A very soft coagulum is formed.

Gelatine is liquefied in less than 24 hours.

Inspissated serum. The growth on this medium is fairly plentiful. Digestion begins after a few days' incubation but stops before it has proceeded far owing to the fact that the organism ceases to grow.

Ordinary broth is a good medium.

Action on carbohydrates. A series of serum litmus agar slopes containing various carbohydrates were inoculated and the organism grew

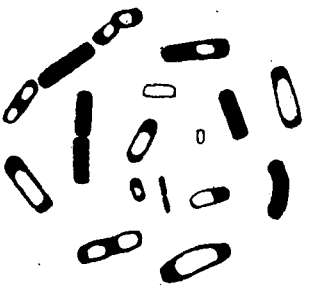

Fig. 2. Representative forms from a two day meat culture.

well. It produced both acid and gas on glucose, maltose and laevulose media, and acid alone on glycerine medium. Neither acid nor gas was produced on dulcite, inulin, mannite, sucrose or lactose media.

On *glucose agar plates*, treated as described for the preceding organism, the growth was very abundant. Pale bluish colonies were formed, usually irregular in outline but with smooth edges. The colonies were entirely homogeneous and of even thickness. The largest ones were two and three millimetres in diameter.

In *deep glucose agar tubes* tiny colonies are formed; they are irregular in outline but they are not radiate nor worm-shaped nor woolly. After five days' incubation they average $\frac{1}{2}$ millimetre in diameter and none of them is more than $\frac{3}{4}$ millimetre in diameter.

Pathogenicity. 2 c.cm. of a 48-hour broth culture failed to produce any pathological symptoms in a guinea-pig when injected into the muscles of the thigh.

TEMPERATURE OF GROWTH. The organism will grow excellently at 37° and also at *c.* 27° but is rather slow in starting to grow at the latter temperature.

AGGLUTINATION. Bacillus II does not agglutinate with the anti-sporogenes serum of Weinberg.

It is of interest to note that the organism bears a close morphological resemblance to *B. perfringens*. Some of its sporing forms are microscopically almost indistinguishable from a violently pathogenic gas forming organism isolated by the author from a human case. This pathogenic organism corresponds closely to vibrion septique, Pasteur.

IDENTIFICATION. I think it is probable that Bacillus II is the same organism as *B. bifermentans sporogenes* of Tissier and Martelly (*Ann. de l'Institut Past.* 1902, p. 894). These authors say however that *B. bifermentans sporogenes* resembles *B. perfringens* and that their rôle seems to be identical. Whether this statement is due to a misunderstanding of the rôle of *B. perfringens*, I do not know, but I can draw no parallel between my organism and *B. perfringens* except the morphological one and the fact that they both split certain sugars. The putrefactive action of *B. bifermentans sporogenes* is far less than that of organism II, the former liquefying gelatine in a month the latter in a night; the former first changing the appearance of milk after five days, the latter showing digestion after a few hours and completely destroying the casein in a few days. These differences are perhaps to be accounted for by different media and technique. Recent experience with anaerobes inclines me to suggest that the strains of Tissier and Martelly were composed of the same organism as mine plus a small proportion of a member of the acid forming group which decreased the proteolytic power of the predominant organism.

Bacillus II resembles morphologically *B. sporogenes foetidus* of Choukewitch (*Ann. de l'Inst. Past.* March and April, 1911) but the latter organism is feebly motile and does not form gas in deep glucose agar.

Clostridium foetidum of Liborius (*Zeitschr. f. Hyg.* I. 1886, p. 160) and *Clostridium foetidum cernis* of Salus (*Archiv f. Hyg.* LI. 118) do not resemble organism II morphologically and they are highly motile.

The "pseudo-oedem bacillus" of Liborius is very meagrely described; it somewhat resembles my organism but is said to form at times two spores in one bacillus.

I wish in conclusion to express my heartiest thanks to Miss Muriel Robertson for her interest in this work and to Dr Harden of the Lister Institute for so kindly affording me the courtesies of the laboratory.