THE PREPARATION OF SUSPENSIONS FOR THE WEIL-FELIX TEST.

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During a period of several months before embarking on his present tour of foreign service the writer devoted himself with some attention to a study of the X strains of Bacillus proteus. Much of the work was merely confirmatory of the work of others, but was considered necessary in order to gain familiarity with the handling of these organisms. The ultimate object in view was the preparation of suspensions for the Weil-Felix test of a kind that would be suitable for distribution to the military laboratories of India.

For the benefit of those who are unacquainted with local conditions it may be said that there are twenty-six military laboratories in India and Burma. The number of specimens examined annually in these laboratories is enormous and increasing year by year. It would be practically impossible for the officers in charge to prepare for themselves the whole of the materials which they require in their routine work. For this reason there was established at Kasauli in 1928 a laboratory, known as the Enteric Laboratory, one of the chief functions of which is the preparation and supply of serums and suspensions for agglutination work. In this manner it acts in the same capacity for India as does the Oxford Standards Laboratory at home, an institution with which the Enteric Laboratory maintains a close liaison.

For purposes of investigation six cultures of proteus were obtained from the Curator of the National Collection of Type Cultures. The tubes were labelled OX2, HX2, OX19, HX19, OXK, and HXK, denoting the "O" and "H" variants of the three types of B. proteus X. As a preliminary, serums were prepared in rabbits from the unselected "H" cultures. At a later stage in the work pure "O" serums were also prepared.

THE FLAGELLAR ANTIGENS OF B. proteus X.

Attention was first directed to the flagellar antigens, and suspensions for "H" agglutinations were prepared in the usual manner as broth cultures sterilized by 0.25 per cent of formalin. Since the motile "H"-agglutinable organisms do not grow as isolated colonies, but as a film spreading over the surface of the medium, colony-selection in order to obtain suspensions of the highest degree of flocculability is unnecessary and
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is even impracticable, except under special conditions of growth. The broth should be inoculated from the spreading growth on a plate, or from the film which rises up the surface of an agar slope when it has been inoculated in the condensation water at the bottom of the tube. If the type of flocculation is not satisfactory, it can be improved by inoculating in the condensation water several agar slopes in succession from the film which rises up the surface of the agar. The sensitiveness of the suspension has not been increased by this method; all that has been done is to improve the flocculability by developing the number of "H"-agglutinatable organisms.

Suspensions prepared in the above manner are incapable of reacting to "O" agglutinin to any appreciable extent within the time of two hours normally allotted for an "H" test. Hence they constitute specific indicators for "H" agglutinin only. In the presence of a very powerful "O" serum some traces of granularity may be visible in low dilution, but this is easily distinguishable from the fat flocculation of which the suspension is capable. Craigie (1931) has given a mechanical explanation of this failure of organisms to show "O" agglutination in the presence of formalized "H" antigen.

A complete series of cross-agglutination and cross-absorption tests was carried out on the "H" antigens. The tests showed that these antigens are mainly identical in the three types, but that minor differences do exist. The difference is very small as between HX2 and HX19, but decidedly more marked as between HXX and the other two types. During the whole of the work on the flagellar antigens no evidence was obtained of the presence of type and group phases, such as are found in the diphasic members of the Salmonella group.

The somatic antigens of Proteus X.

The study of the organisms was then extended to the somatic antigens, a more important matter in view of their connection with the Weil-Felix test. It has been said that three of the cultures obtained from the National Collection of Type Cultures were labelled OX2, OX19, and OXK. Whatever was the condition of these cultures on receipt, it was quickly apparent, when they were examined a fortnight or so later, that in the case of OX19 and OXK the labels had become misnomers. These two cultures had reverted to the "H" form and differed in no way, either in the characteristics of their growth or in their agglutinating properties, from HX19 and HXK.

The matter was otherwise, however, with OX2. Felix (1933) has shown that this particular strain is extremely stable in the "O" form. This finding was confirmed, for at no time in the hands of the writer did this culture show any signs of "O-H" reversion, in spite of the most ardent and prolonged coaxing to induce it to do so.

Since OX19 and OXK had become HX19 and HXK, it became necessary to isolate the "O" variants afresh. This is a simple matter but may take
some days to accomplish. The culture is thinly plated on litmus lactose agar, and after twenty-four hours' growth it will be found that, although the whole surface of the plate may be covered with filmy growth, yet spots are apparent where individual bacilli started to grow into a colony before becoming submerged in the swarm. The point of a platinum needle is lightly touched into the summit of one of these spots and the growth so removed replated. On the following day the amount of filmy growth will be found to be appreciably less than on the first plate, and individual colonies more evident. The same procedure is carried on from day to day, until after five or more such platings isolated colonies are obtained which are entirely purified from filmy growth. A test will now show—one feels it might be safer to write "may show"—that the culture is completely devoid of "H" antigen.

Another method which may be tried is to plate the organism on phenol-agar (1:1,500). On this medium discrete colonies are obtained and a number are picked on to agar slopes. After incubation for twenty-four hours it may be observed that on some of the slopes there is less tendency to the formation of filmy growth than on others. By further selection in the same manner a pure "O" strain may at length be obtained.

A point of some importance must be mentioned here. It is often difficult to decide by the appearance of the agglutination whether this is of the "H" or "O" variety. Fortunately the organisms themselves provide a ready means of differentiation; or rather, it would be more correct to say, of determining whether "H" antigen, capable of being agglutinated, is present in the suspension or not. Since the organisms have flagellar antigens in common, but differ in their somatic antigens, all that is required is to test the suspension with the "H" serum of one of the heterologous types. If well marked agglutination occurs rapidly it must of necessity be of the "H" variety, thus proving that "H" antigen is present in the suspension.

During the investigation of the "O" antigens some degree of cross-agglutination was observed between the three types. This was regarded as normal when occurring between OX2 and OX19, since a common somatic factor is known to exist between these organisms. Cross-agglutination was also observed between the highly stable OX2 culture and HXK serum, and might reach as high as 25 or 50 per cent of the serum titre. But in this case the agglutination was of an incomplete type; a few of the organisms only were agglutinated, the majority remaining in stable suspension. On the other hand it was found that if the "O" variant was isolated from HX2 culture and tested with HX2 serum this form of cross-agglutination was not obtained. Moreover, it was found that "O" (HX2), as the variant derived from HX2 may be named, was always agglutinated to a higher titre by HX2 serum than was OX2. These facts led one to believe that the complete stability of OX2 in the "O" form must be due to some rough or other degenerative change in the antigen.
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THE "O" VARIANT.

The question had now to be considered as to the best form of material to supply to military laboratories in India for use in the Weil-Felix test. Felix (1933) has recommended the use of the living "O" variant in the test, and has described the manner in which it may be maintained in pure condition. The essential feature of this method is the use of dry agar as a culture medium. The possibility of distributing living cultures of the "O" variant was considered, for there is no doubt that, when pure, it is the most sensitive reagent that can be employed. But the tendency of the "O" variant to revert to the "H" form has already been noticed, and it constitutes a serious disadvantage in its use; for the presence of a trace of "H" antigen, even in a living suspension, markedly lowers its sensitivity to "O" agglutinin. So important is this matter that in one's own work it has become the rule always to test the living suspension for the presence of "H" antigen in the manner previously described, before making use of it in the test.

For the above reasons it was felt that the use of the living "O" variant was unsuited to conditions in India. As we have seen, officers in charge of laboratories are occupied with an enormous amount of bacteriological and biochemical work, and to burden them further with the necessity of keeping a constant watch on the purity of their proteus "O" cultures was not practical politics. It seemed, therefore, that the provision of a ready-made killed suspension was clearly indicated, even though this might be somewhat less sensitive than the living culture.

CONCENTRATED SUSPENSIONS.

For some time one had been impressed by the advantages afforded in all agglutination work by the use of suspensions in concentrated form; instead of the usual fifteen drops which are added to each tube in a Dreyer's test, one drop only is required, together with fourteen drops, or their equivalent in volume, of physiological saline solution. So far as a central distributing laboratory is concerned, among the advantages arising from the supply of such material may be stressed the greatly diminished cost of packing and postage, no mean advantage in times like the present.

But there are advantages also in practical application. The use of a concentrated suspension enables an agglutination test to be set up in a minimum of time by eliminating much of the drop-counting, so tedious when many serums are being examined. After addition of serum (10, 5, 2, 1, etc., drops in the ordinary way) to all tubes, these are filled up rapidly to about half an inch from the brim, and one drop of suspension is then added on the top. It may be objected that this method must give rise to a considerable error owing to variations in bore of agglutination tubes. With a normal set of tubes this error would be slight in any case; but experiment proves that the error in reading shown in any particular tube is not in proportion to, but is actually much less than, the error in dilution.
which may be present. The chief factors which determine whether or not agglutination will occur in any particular tube are the actual quantity of serum present in that tube and the number of agglutinable organisms on which that serum will react. The result is largely independent of the total quantity of fluid in which the two reagents may happen to be floating. "Dilutions" afford a convenient method for setting up the test and reporting its results, but they have not much significance beyond that.

**Possible Methods of Preparation.**

In the preparation of a killed "O" sensitive suspension from any motile organism several possible methods are available. The "O" variant, isolated by selection from the "H" culture, may be used in the form of a formolized broth culture; or the "O" variant can be grown on agar and washed off into saline containing some reagent, usually formalin, which will kill and preserve the bacilli. Since it was hoped to prepare suspensions in concentrated form, the use of broth cultures was ruled out of court. In both methods it is necessary to ensure that the cultures are completely devoid of "H" antigen, since the effect of formalin on the sensitiveness of the "O" antigen in the presence of "H" antigen has already been referred to. The difficulties which may be encountered in growing the "O" variant in its pure condition have been suggested. These difficulties are in no way lessened when it is proposed to inoculate large surfaces of agar for the mass production of agglutinating material. The need for dry agar has been mentioned; but it is not always possible to ensure that the agar shall be of a suitable degree of dryness on the particular day it is required. The presence of a little moisture may ruin a whole batch.

Growth on phenol-agar (1:800) for the suppression of the "H" antigen was tried, for on this medium, as we have seen, the "H" culture grows in the form of discrete colonies with no suggestion of filmy growth. But appearances were deceptive. Tests showed that "H" antigen might still be present in significant amount.

There remained the methods by which the "H" antigen is destroyed, at least so far as its power to agglutinate is concerned. This can be effected either by heat or by treatment with alcohol. In the case of the *proteus* strains the application of heat by boiling rendered the organisms auto-agglutinable, and the more they were boiled the more auto-agglutinable they became. No success attended this method.

Alcohol then was the last resort. It may be applied in one of two ways: by the method of Bien, quoted by Gardner (1931), in which the final concentration of alcohol in the suspension is 33 per cent. This method was not found suitable for the preparation of concentrated suspensions. In the first place, the large amount of alcohol present in the suspension markedly diminishes the size of a drop delivered by a pipette; and in the second place, the drop tends to remain floating on the top of
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the serum-saline mixture in the tube. The two fluids must then be mechanically mixed, causing irritating delays.

In the second method alcohol is applied in more drastic fashion. It is added to the saline suspension in excess, and is subsequently removed by decantation and centrifugalization. The organisms are then resuspended in physiological saline solution. From the first this method gave better results than any other, and it was well adapted for the production of suspensions in concentrated form.

One other matter needed consideration—the question of the preservative; for it was found that the resuspended organisms were inclined to become contaminated, unless something was added to prevent this. A number of preservatives was tried, but none was found so satisfactory as formalin. And here a valuable suggestion was received from Dr. Gardner of the Standards Laboratory, Oxford. It is known that formalin-preserved suspensions may be of good quality when first prepared, but tend to deteriorate owing to acidity resulting from disintegration of the formalin. Dr. Gardner suggested that this trouble might be overcome by buffering the formol-saline solution to a pH of 7.6 by means of disodium hydrogen phosphate.

There was no opportunity of putting this suggestion to practical test before the writer received his final embarkation orders. Packing up the proteus strains in his "not-wanted-on-the-voyage" kit, he departed for India, where in due course he took over charge of the Enteric Laboratory. It must not be thought, however, that in the immediately preceding years the Enteric Laboratory had been behindhand in the provision of materials for the Weil-Felix test. Alcoholized suspensions of several proteus strains had been prepared in dilute form for some years and regularly supplied to laboratories inquiring for them.

Selection of Cultures and Colonies.

In the Enteric Laboratory at this time a number of strains of proteus X were available to choose from, and it was found that they differed somewhat in the liveliness of their response to "O" agglutinin. Of type X19 there were the Muktesar and Warsaw strains referred to in a previous paper (1934), both of them in the "H" form. There were also the OX19 (?) and HX19 which had been brought out from home. Tests showed that of these strains the Muktesar culture was the most sensitive to "O" agglutinin, and it has therefore been used throughout for the preparation of suspensions. Of type XK there was a strain which had been obtained from Malaya some years previously, together with the OXK and HXK which had been brought out from home. Of these the Malay strain gave the best results. In the case of type X2 there were only available the OX2 and HX2 brought from home. For reasons already given OX2 was regarded as an unsuitable culture to use.

Although the difference is not very great, it has been commonly found
that in the alcoholizing process the "H" culture has given better results and has produced a more highly sensitive suspension than the pure or semi-pure "O" variant. The reason for this is not clear, but it may be due to the fact that the "O" variant has already been subjected to a process of selection, not however with a view to obtaining the most sensitive colonies, but solely with the object of isolating those organisms in which the power to develop "H" antigen has been suppressed. It is possible that during this process the less sensitive colonies may have been picked. However that may be, the fact that "H" cultures give better results absolves one from the necessity of trying to maintain "O" variants in pure conditions, or indeed of keeping them at all.

If it is desired to produce suspensions of the highest possible degree of sensitiveness, showing rapid and clean agglutination with a sharp endpoint, it is advisable to prepare them from a single selected colony. On the other hand, if time is short or if one is satisfied with suspensions of a somewhat inferior quality, then this procedure of colony-selection may be omitted, and the suspensions prepared in blunderbuss fashion from the whole culture.

NOTES ON TECHNIQUE.

The technique which was ultimately adopted for the preparation of concentrated alcoholized suspensions is given in Appendix I.

Some notes will now be made on the method.

Alcohol.—It will be seen that, both in the preliminary test for selection of colonies and in the final process, the amount of alcohol used is rather large, and indeed seemed likely to become somewhat a strain on the financial resources of the laboratory. For this reason methylated spirit was tried in its place and found to be quite as effective as the purest alcohol available. Methylated spirit is, of course, much cheaper than alcohol, and, in fact, costs the laboratory nothing, being a "free issue" to laboratories in India by the Indian Army Service Corps. The spirit should be filtered quite clear of deposit or opalescence before use.

Resuspension of the Organisms.—It is inadvisable to resuspend the organisms direct in a formol-saline solution, for if this be done great difficulty may be experienced in smoothing out the clumps and obtaining a suspension entirely free from granularity. A measured quantity of sterile saline should be added first. Then, after the material has been transferred to bottles and vigorously shaken, 2 per cent buffered formol-saline solution is added in the proportion of one part to seven parts of suspension. This gives a final concentration of formalin of 0.25 per cent.

Standardization.—When one drop of suspension of a concentration equivalent to 6,700 million B. coli per cubic centimetre is added to an agglutination tube filled with saline, the resulting opacity in the tube is rather greater than that commonly employed in an agglutination test. But it is thought that with this rather greater density of organisms the inter-
mediate readings between "total" and "trace" are more readily perceived.

Standardization should be carried out with care, since it affects the sensitiveness of the suspension. Some not very exact tests showed that if the density of the suspension is reduced to a half, its sensitiveness is increased by about 40 per cent; and if density is reduced to a third, then sensitiveness is increased by about 80 per cent. These figures are only approximate, but they are sufficiently accurate to show that, in contrasting the sensitiveness of any two suspensions the question of their comparative densities is a matter of considerable importance; and it is one that is commonly overlooked.

In practice the following method of standardization is adopted. Tube 3 in Brown's series only is used, since it is thought to be more easily-matched than any other. On the scale in use this tube has a value of 1,230 million B. coli per cubic centimetre. Hence the density required in the suspension is 5.5 times the value of tube 3. One volume of suspension is diluted with volumes of saline until it is found to match tube 3. Then the amount of fluid which must be added to bring the suspension to the required density is calculated from the following formula:

\[
\frac{(a - 5.5) x}{5.5}
\]

where "a" is the number of times the suspension must be diluted to bring it to the value of tube 3, and \( x \) is the volume of suspension to be diluted. Thus, supposing we have 50 cubic centimetres of suspension and it is found that it must be diluted with eleven volumes of saline, or twelve times, to bring it to the density of tube 3, then the quantity of fluid which must be added to give a concentration equivalent to 6,700 million B. coli per cubic centimetre is equal to 6.5 \( \times \) 50 \( \div \) 5.5, or 59.1 cubic centimetres. This fluid is added as to seven-eighths in the form of sterile saline and one-eighth of 2 per cent buffered formol-saline. The concentration of formalin is thus retained at 0.25 per cent.

**Incubation.**—Felix and Olitski (1929) have shown that the temperature of incubation should not be too high, since the agglutinin in a typhus serum is markedly heat-labile. When using alcoholized suspensions a temperature of 50° to 52° C. in the water bath for four hours followed by all night in the 37°C. incubator gives good results. On the following morning the racks should be returned to the water bath for a few minutes before reading the test. This freshens up the tubes and renders the end-point more decisive.

Incubation throughout at 37°C. is also satisfactory, but the higher temperature has the advantage that within an hour one can see whether the test will be positive or negative, and obtain a good idea of what the end-point will be.

**Reading the test.**—A scheme for determining the point of standard agglutination is given in Appendix II. This is based on Dreyer's reduction...
table (1920) with the figures reduced to round numbers. It is felt that those who do not make use of interpolation figures of this kind, but are content to read their tests merely to the dilution-value of the tube in which the end-point occurs, are rejecting much information of value that the test can afford.

Summary.

Concentrated alcoholized suspensions prepared in the manner described are believed to have given good results throughout India, and to have been the means whereby many cases have been diagnosed typhus fever, which otherwise might have been classed as enteric group, etc. These suspensions retain their agglutinating properties unimpaired during storage for at least six months; and they do not become contaminated except under conditions of gross ill-usage.

It is true that living suspensions of the "O" variant are more sensitive, to the extent of perhaps 50 per cent, but one would not regard them as superior reagents for that reason alone. They may be recommended to those who have the time and experience necessary to use them with discretion, but they are considered unsuitable for routine use in busy laboratories; neither are they suitable for those laboratories in which it is only required to carry out a test occasionally. In such cases certainty and uniformity are thought to be more desirable qualities than a very high degree of sensitiveness.

Appendix I.

Technique of preparation.

1. Plate the culture to be used on phenol agar (1:1,500) to ensure growth in single colonies. Incubate twenty-four hours.

2. Pick a number of colonies, say six, each on to two agar slopes, and incubate for twenty-four hours. The one slope is used for test, the other is kept as "office copy."

3. Add about two cubic centimetres of saline to each test slope and wash off the growth.

4. Pour off the suspensions into clean tubes and fill the tubes about three-quarters full with alcohol. Shake up all the tubes and put them aside for twenty-four hours.

5. Pipette off as much as possible of the clear supernatant alcohol, leaving the deposited organisms. Fill the tubes half full with saline and shake up thoroughly. Dilute further with saline and reduce to suitable density for agglutination test.

6. Test all suspensions with type serum in a series of dilutions depending on the titre of the serum. Choose that suspension which agglutinates most rapidly, most completely, and to the highest titre.

7. Growth in bulk is carried out in Roux bottles or screw-capped "medical flats" (McCartney 1933) which have been coated on one side with unfiltered agar. Pour into each bottle the contents of one broth tube which has been inoculated rather heavily from the office copy of the selected colony. Allow the broth to
flow over the whole surface of the agar. Incubate for twenty-four hours with the bottle on a slope so that the broth remains at one end.

(8) Add a small quantity of saline to each bottle and wash off the growth. Filter through cotton wool into narrow-mouthed bottles. Add alcohol in the proportion of not less than four volumes to one volume of suspension. Tightly cork the bottles and shake up thoroughly several times. Stand the bottles aside for twenty-four hours.

(9) Pour off as much as possible of the supernatant alcohol and transfer the remainder containing the organisms to centrifuge tubes. Swing rapidly for a few minutes. Pour and pipette off the whole of the alcohol.

(10) Grind up the organisms in sterile saline and transfer to sterile narrow-mouthed bottles. Tightly cork the bottles and shake very thoroughly until it is seen that all clumps have been smoothed out and no trace of granularity remains.

(11) Add 2 per cent buffered formol-saline to make the concentration of formalin 0.25 per cent.

(12) Standardize the suspension by adding more sterile saline and 2 per cent buffered formol-saline (final concentration of formalin 0.25 per cent) to a density equivalent to 6,700 million B. coli per cubic centimetre.

APPENDIX II.

SCHEME FOR DETERMINING THE POINT OF STANDARD AGGLUTINATION.

(Based on Dreyer's reduction table.)

| Trace minus | 25 | 50 | 125 | 250 | 500 | 1,000 | 2,500 | 5,000 |
| Trace       | 15 | 30 | 75  | 150 | 300 | 600   | 1,500 | 3,000 |
| Trace plus  | 20 | 40 | 100 | 200 | 400 | 800   | 2,000 | 4,000 |
| Standard minus | 22 | 45 | 110 | 235 | 450 | 900   | 2,250 | 4,500 |
| Standard    | 25 | 50 | 125 | 250 | 500 | 1,000 | 2,500 | 5,000 |
| Standard plus | 27 | 55 | 135 | 275 | 550 | 1,100 | 2,750 | 5,500 |
| Total minus | 30 | 60 | 150 | 300 | 600 | 1,200 | 3,000 | 6,000 |
| Total       | 35 | 70 | 175 | 350 | 700 | 1,400 | 3,500 | 7,000 |

REFERENCES.