BACTERIUM TYPHOSUM.

The Development of Vi-Antigen and Vi-Antibody.

By MAJOR H. J. BENSTED, M.C.,
Royal Army Medical Corps.
(The Enteric Laboratory, Kasauli.)

In a previous communication (Bensted, 1937) a report was made on the laboratory findings in a series of cases of typhoid fever. The report was chiefly concerned with the mouse virulence and Vi-antigen content of the organism recovered from the blood culture and the findings, which were in general agreement with those of other workers, were fully confirmed in the following year in a similar, but more extensive, report by Lewin (1938).

The present communication is concerned with the examination of a further series of strains of Bact. typhosum, chiefly with regard to the enhanced development of Vi-antigen, and also an investigation into the production of Vi-antibody in natural infections in man, induced infections in animals and the response following the injections of killed suspensions of certain Vi-strains.

Vi-content of Freshly Isolated Strains and the Change Induced by Artificial Culture Media.

The antigenic analysis of a further 380 recently isolated strains has since been carried out, and out of this number only four were encountered that appeared to be completely devoid of Vi-antigen. Whilst the remaining 376 cultures were all rich in Vi-antigen there were only 18 that were fully resistant to "0"-agglutination when originally examined. In the report previously communicated 16 per cent of the strains were designated as pure V-forms. At that time, however, it was not fully appreciated the extent to which resistance to "O"-antibody may be influenced by the period of incubation of the cultures. Strains containing minimal amounts of Vi-antigen may appear to be completely "O"-resistant after five or six hours' incubation, whereas after twenty-four hours or more it may be difficult to appreciate more than a trace of Vi and the culture appears to be fully sensitive to "O"-antibody. True Vi-strains remain "O"-resistant after much longer periods of incubation, even when grown on ordinary digest-agar and stock cultures of such strains as Ty. 2, Watson, Raw-Ben, etc., that have been maintained on egg-media, for still longer periods. Growth scraped from the surface of month-old cultures on this medium is fully sensitive to a pure Vi-serum and shows but slight reaction to "O"-antibody. Table I shows the difference in "O"-resistance, according to the length of
incubation, between classical Vi-strains and freshly isolated cultures under test:

**Table I.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Age of culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 hours</td>
</tr>
<tr>
<td></td>
<td>Vi</td>
</tr>
<tr>
<td>Ty. 2</td>
<td>++</td>
</tr>
<tr>
<td>Raw-Ben</td>
<td>++</td>
</tr>
<tr>
<td>T. 240..</td>
<td>++</td>
</tr>
<tr>
<td>T. 271..</td>
<td>++</td>
</tr>
</tbody>
</table>

Amongst the cultures examined there was a batch of 12 that originated from an isolated epidemic in a boarding school where all the pupils were reported fully protected by T.A.B. vaccine. The cases were very severe and two were fatal, yet there was the widest difference in the Vi-content of the cultures recovered from the blood—more marked than Perry, Findlay, and Bensted (1933) found in the Malton outbreak of 1932, even though all cultures from the latter were from fecal isolations. In the epidemic in question cultures from one fatal and one other case appeared to be completely devoid of Vi-antigen, nine contained Vi, but were very sensitive to "O"-antibody, whilst only one was "O"-resistant.

The value of egg-media in the maintenance of Vi-antigen has been appreciated for some time (Kauffmann 1935, Bensted 1937, Felix 1938), and has been employed for that purpose by the present writer since 1933, but this medium would, under certain conditions, appear to have the further power of enhancing the development of the antigen. In January, 1935, a number of typhoid strains were subcultured on to egg-medium in small MacCartney bottles. After the preliminary opening a few weeks later, for examination, several of them remained sealed until the beginning of 1938. Those of special interest were: "T.901 OH," "T.901 O," and the original rough Rawlings, and after three years in a stock-culture box, subjected to a great range of temperatures, between 40° and 120° F., they were subcultured on to digest-agar and good growths were obtained after twenty-four hours' incubation.

The old Rawlings strain, which originally contained only a minimum amount of Vi and had an A.L.D. for mice in the region of 400 millions, was now completely "O"-resistant whilst responding very actively to a pure Vi-serum and its A.L.D. for mice was between 50 and 80 million.

Vi-forms have been recovered from "T.901 OH" strains by other workers (Craigie and Yen, 1938), but this was not appreciated when similar results were observed in this laboratory and even more striking was the demonstration of very definite amounts of Vi-antigen in the field cultures of "T.901 O." (This was enhanced after colony selection, but was sufficiently obvious in young field cultures as to put all doubt aside.)
In a previous communication (1937) it was stated that two series of Vi-strains, one "O"-resistant and the other "O"-sensitive, had been seeded on to egg-media and the bottles sealed and set aside for examination at a later date. They have recently been examined after an interval of three years: all the "O"-resistant forms were found to have retained their original properties and Table II illustrates the change that took place in the other series.

Table II.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Result in 1936 with</th>
<th>Result in 1939</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pure Vi-serum</td>
<td>Pure &quot;O&quot;-serum</td>
</tr>
<tr>
<td>V.W.1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>V.W.2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>V.W.3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>V.W.4</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>V.W.5</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>V.W.6</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

It will be seen that one strain has remained "O"-sensitive. This has been noted but rarely. Reversion of stock Vi-strains occasionally takes place also. For instance two one-year-old cultures of Raw-Ben were found to have become "O"-sensitive, yet the original three-year-old stock yielded cultures that were completely "O"-resistant.

Except where stated, colony selection was not employed in any of the work described above; any change in the antigenic structure described was demonstrated in the field-cultures of that particular strain.

In the early part of 1938 Major Bhatnagar kindly sent the writer a subculture of a strain of Bact. typhosum that he had received from Europe. This strain, now known as Vi.1, was so deficient in "H" and "O" antigens that suspensions prepared from agar-washings were sensitive only to Vi-antibody. The properties of this variant have been fully described by Bhatnagar (1938), who particularly draws attention to its rough tendency. Nevertheless, in spite of the rough appearance of the colonies, suspensions can be readily prepared that are stable in high concentrations of saline, but, constant care must be exercised in subculturing the strain, as is the case with all Vi-stock cultures, to ensure that the new growth retains the full sensitivity and specificity of the parent stock.

The serological behaviour of the new Rawlings strain, known now as "T.RV," appeared to be so similar to this Vi.1 strain (the early subcultures failed to react with "H"-antibody) that it was hoped that the two antigens
Bacterium Typhosum

could be used in parallel for the estimation of Vi-antibody, by a simple
direct method. When, however, bulk quantities of "T.RV"-suspensions
were prepared it was found that small amounts of "H"-antigen were
invariably present and it was, therefore, decided to use the "T.Vi. 1" alone
for the time being. The suspensions of the latter organism were prepared
by washing off the eighteen hours' growth from saccharose-digest-agar in
1:5,000 mercuric iodide. The saccharose medium gives an excellent
growth of bacteria and, although not obviously superior to ordinary digest­
agar in the case of other Vi-strains, does appear to be of definite advantage
with this particular strain.

The thick creamy washings from as many Roux bottles as are considered
necessary are first strained through packed cotton-wool and then diluted
down by the addition of further quantities of mercuric iodide to the standard
opacity of the "concentrated agglutinable suspension" described by Bridges
(1935) and bottled in 5-c.c quantities. This suspension was given extensive
trials in this laboratory and the results were considered sufficiently satis­
factory to warrant an experimental issue to a few other laboratories. It was
at first prepared in small batches and issued for immediate use only after
its agglutiniability and specificity, etc., had been tested.

Meanwhile efforts were made to produce a more satisfactory "T.RV "-suspension. Bien and Sonntag (1917) had found that the addition of alcohol
to saline suspensions of the "X"-strains of the proteus organisms did not
reduce their agglutinability markedly, and they noted that such suspensions
retained their properties over long periods. The explanation of the success
of this procedure was, of course, partly due to the sterilizing properties of
alcohol and partly due to its action, as Craigie (1931) showed, in removing
the free part of the flagella so that the non-specific "H"-agglutination did
not interfere with the reading of the Weil-Felix reaction. A modification
of Bein and Sonntag's technique has been in use for a number of years in
this laboratory for the production of pure "O"-suspensions (Bridges, 1935).
Felix and Pitt (1934) remarked on the apparent destruction of Vi-antigen
by alcohol and recently Felix (1938) stated that whilst alcoholized suspensions
of Vi-strains were suitable for the production of Vi-antibody, yet they were
insensitive to the action of Vi-agglutinins. This had been our own experience
with many strains, but it had been observed that some cultures, even after
prolonged contact with alcohol, continued to react with a pure Vi-serum.
The original alcoholizing process for the production of "O"-suspensions
lasted for twenty-four hours, but recently this period has been very much
reduced and at the present time the suspension is shaken in alcohol for
half an hour only. It was whilst experimenting with the reduction in time
of the alcoholizing process that the stability of certain Vi-cultures towards
alcohol was noted. "T.RV" and "T.Vi. 1" especially, after several hours
in the presence of alcohol, failed to show any reaction with a pure "O" serum. Nevertheless, long contact with alcohol tended to produce suspen­sions that became granular and auto-agglutinable and occasionally reduced
the Vi-sensitivity and, therefore, as five or ten minutes' shaking with alcohol produced a suspension (in the case of sluggishly motile bacilli) that completely failed to react with a pure "H"-serum, there was everything to be gained by the shorter contact. Also by killing the bacteria very quickly in mercuric iodide before shaking with alcohol the loss of Vi-sensitivity appeared to be reduced to a minimum. It was found then that a pure Vi-suspension of the "RV"-strain could be prepared by washing off the eighteen to twenty hours' growth from digest-agar with 1:5,000 mercuric iodide, after about one hour's contact four times the bulk of alcohol is added and the mixture shaken very frequently during the next five minutes. Flocculation takes place rapidly, and generally in less than another five minutes the supernatant alcohol can be removed by a suction pipette. The residue is then centrifugalized rapidly, and the centrifugalate, finally drained of alcohol, is re-suspended in 1:10,000 mercuric iodide and the opacity standardized to that of the "concentrated agglutinable suspension."

Suspensions prepared in the manner described were found to be as sensitive to Vi-antibody as those of T.Vi.1, yet unaffected by pure "O" or "H" sera, and to be completely stable in all concentrations of saline up to 6.8 per cent.

**Estimation of Vi-antibody.**

Although in the communications of Felix (1938) and Bhatnagar (1938) certain details of method are given, it is thought that a description of the technique employed in this and other military laboratories in India for the past year and more might be of interest.

The reagents employed are the two Vi-suspensions, "T.Vi.1" and "T.RV" described above, and a standardized Vi-serum is also issued in order that Vi-agglutination may be properly appreciated and comparable results obtained. The Vi-serum prepared in this laboratory is very carefully compared with the "provisional standard Vi-serum," kindly supplied by Dr. Felix, by carrying out parallel tests with several batches of "T.Vi.1" and "T.RV" and also Watson suspensions. Unless it gives results of the same order it is not issued. Felix's serum is prepared from the horse and is unpreserved, whereas the serum prepared in this laboratory is a glycerinated rabbit serum. Unpreserved serum is not suitable for general issue in this country owing to the extreme changes of temperature and the ease with which contaminations occur. Glycerinated Vi-serum, prepared over four years ago by the present writer, has retained its original titre although subjected to rough usage and a great range of temperatures.

The agglutination tubes are round-bottom test tubes, 2 by \(\frac{1}{2}\) in., and they are arranged in low wooden racks so that each serum can be tested against four antigens, the two Vi-suspensions mentioned above, and also pure "O"-antigens of Bact. typhosum and Bact. paratyphosum A with which this communication is not directly concerned.
**Bacterium Typhosum**

The serum dilutions are carried out by the "doubling dilution" method by means of a volume pipette of the type shown in the diagram below.

The pipette has a capacity of about 0.75 c.c. and is used with a large half-ounce bulb teat that fits comfortably into the half-closed hand. With a little practice long series of accurate deliveries can be made quickly and without fatigue.

In view of the low dilutions employed in the first tube of any series it is essential that the serum should be bright, clear, and entirely free from haemoglobin.

For the test one volume of saline is first introduced into every tube set out, then one volume of the serum, diluted two and a half times, is pipetted into the first tube of each row. The double volume in the first tube is well mixed by sucking up and down twice in the pipette and then one volume is transferred to the second tube, and so on to the last tube but one in each row. The actual serum dilutions are therefore 1:5, 1:10, 1:20, etc., and the last tube, which contains no serum, is the control. The concentrated agglutinable suspensions are then introduced by means of a Dreyer standard dropper—one drop into each tube, which represents about one-thirtieth of the final bulk. The rack is shaken as in the Kahn flocculation test, but only for fifteen seconds, and placed in the incubator at 37° C. for two hours, after which it is removed and allowed to remain at standard room temperature until the next morning, when the results are read. As the temperature in several laboratories in India may be over 100° F. for many hours during the day the cool chamber is recommended in such circumstances and has been found to give satisfactory results.

The reading of the test requires considerable care and experience. The first essentials are that the lowest dilutions should show total agglutination with the supernatant fluid clear and that the negative controls should show the unagglutinated bacteria agglomerated in a central mass at the bottom of the tube. The actual end-point, as stated by Felix (1938), is that dilution of serum that produces Vi-agglutination with the Vi-suspensions issued to a degree equal to that given with the titred standardized Vi-serum.

**Development of Vi-antibody.**

*In Individuals Suffering from Typhoid Fever.*—The presence of Vi-antibody in the sera of typhoid patients was recorded by Felix and others (1935), Gundel and Abdoosh (1936), Bensted (1937), and several other workers, but as the demonstration had generally involved the preliminary
absorption of the "H" and "O" antibodies it was hardly a practical proposition for routine work, and as the absorbing agent generally used was "T.901 OH," which frequently contains Vi-antigen also, the estimations would tend to lack accuracy. It is true that Felix employs a direct method that gives excellent results in his hands, but it does not appear to have been used largely by other workers.

With the introduction of killed suspensions that were sensitive only to Vi-antibody it was hoped that its titration might be carried out with reasonable accuracy by the average laboratory worker. A scheme was evolved to enable these determinations to be carried out by a number of laboratories in India, and they were supplied with the materials mentioned in the previous section and also very full instructions. The instructions were supplemented by numerous personal communications and also by the frequent exchange of test sera to ensure the correct standard was being adopted for the reading of the results.

The early results were irregular and of only partial value owing to the inexperience of many of the workers with regard to the new technique and the failure to appreciate the necessity for observing total agglutination in the low dilutions before accepting Vi-agglutination. However, as soon as the standardized serum was taken into general use and its value appreciated the majority of laboratories—as judged from the results of the exchange of sera—were obtaining comparable readings. There was complete agreement about a negative result but there was a tendency to read Vi-agglutination a step too high. This is mentioned in connexion with the report below, which concerns certain Vi-estimations carried out in other laboratories.

Bhatnagar (1938) recorded a series of cases of typhoid fever in which he had found Vi-antibody production very regular. In every case where the causative organism had been recovered from the patient he was able to demonstrate appreciable amounts of Vi-antibody in the blood serum. On the other hand Pijper and Crocker (1939), although employing the method involving the preliminary absorption of "H" and "O" antibody, found the development of Vi-antibody very irregular, and in three out of eight control cases were unable to demonstrate its presence at any time during the illness.

The present report is concerned with the investigation of Vi-development in eighty cases of typhoid fever where the diagnosis was confirmed by the cultivation of Bact. typhi from the blood of the patient. Although a certain amount of the work was carried out in other laboratories the bulk of it was either confirmed or carried out personally.

In 19 out of the 80 patients there was a complete absence of Vi-agglutination in serum dilutions of 1:20 upwards throughout the disease. Of the 19 individuals six were tested at frequent intervals from the third day of onset, whilst in the remainder the first examination was not carried out until the seventh day or a little later. Vi-antibody was demonstrated in the blood-serum of the remaining 61 patients. Appreciable titres were
observed in 18 cases before the end of the first week, and in the remaining 43, the first positive agglutination result at varying times between the eighth and twenty-first day.

19 patients failed to show any Vi-antibody.
19 patients showed a maximum titre between 5-40.
36 " " " " " " " " 80-160.
6 " " " " " " " " above 200.

The Vi-agglutinin curve does not appear to follow regular lines, but in 23 patients the following types were encountered:

In one of the positive cases the titre was 1:20 on the seventh day of illness although blood cultures taken on the fifth, seventh, and tenth failed to grow any organisms. The clinical picture for ten to twelve days was that of an almost symptomless pyrexia, but in the third week the patient's condition steadily deteriorated and the picture was typical of enteric. Further blood cultures resulted in profuse growths of *Bact. typhosum*. Although similar cases are met from time to time this is the only occasion on which there has been an opportunity of demonstrating Vi-antibody before the recovery of the organism from the blood has been possible.

With regard to the thirteen cases, examined at intervals after the seventh day without showing the presence of Vi-agglutinins, it is possible that demonstration of this antibody might have been effected had earlier samples of serum been available. Two positive cases had shown appreciable titres, 80 and 160 respectively, before the end of the first week, but by the tenth day the titres had fallen to below 5.
Only 10 of the negative cases were regularly tested in dilutions as low as 1:5, and as the titre of one of the positive cases never rose above that figure it is possible that lower dilutions in all the examinations might have disclosed a higher proportion of Vi-agglutinins.

Actually there were five cases, tested in dilutions of 1:5 upwards from the third day of onset, that failed to show any Vi-agglutinins. In spite, therefore, of the reservations mentioned above, it would appear that the development of Vi-antibody during an attack of typhoid fever may be so slight that the usual methods employed for its demonstration may fail to establish its presence.

In Convalescent Carriers.—Four cases were encountered where the excretion of the typhoid bacillus persisted for more than one month after convalescence was complete. Two of these cases showed a secondary rise in the Vi-titre during convalescence and special precautions were taken in the examination of the stools and urine which resulted in the recovery of Bact. typhosum from the stool in each case. The Vi-titre rose to 80 in the first case and remained at that height during the carrying period. The other showed a steady rise throughout and the titre eventually reached just over 700 (the highest figure observed personally in human serum). Two months after the carrying period was over the Vi-titre was 20.

There was another patient who during convalescence showed a definite rise in Vi-titre from zero in steps up to 160, beginning two months after the onset of the disease and nearly three weeks after full convalescence had set in. But in spite of the very complete examination of over 60 specimens of urine and faeces by both direct and enrichment methods it was not possible to demonstrate a carrier condition.

The other temporary carriers failed to show any Vi-antibody in their sera at any time during which they were excreting Bact. typhosum, although it was present in low titre during the early part of the illness.

In Chronic Carriers.—During the routine examination of the stools and urine of healthy individuals for carrier conditions seven were found to be excreting Bact. typhosum. In six cases the organism was found to be present in considerable numbers in every stool passed, but in the seventh case typhoid bacilli were only found on one occasion. Blood-serum from the individuals in question was examined for Vi-antibody in dilutions from 1:5 upwards as recommended by Felix (1938), and the results are summarized below:—

<table>
<thead>
<tr>
<th>Number of individuals</th>
<th>Vi-titre of serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Nil</td>
</tr>
<tr>
<td>1</td>
<td>1-5</td>
</tr>
<tr>
<td>3</td>
<td>1-10</td>
</tr>
<tr>
<td>2</td>
<td>1-40</td>
</tr>
</tbody>
</table>

The above figures were all confirmed personally. The serum that failed to show the presence of any Vi-antibody was from the individual who only
showed *Bacterium Typhosum* in the stools on one occasion. Only three examinations were carried out as the man moved from the district and it was not possible to trace him. Other carriers were reported without Vi-antibody in their serum, but as low dilutions had not been employed in the test they have not been included in this series.

Considerable interest attaches itself to one of the cases. A female servant was found to be a fecal carrier in 1937. Record of her was lost until the early spring of 1939, when her stools regularly contained very large numbers of *Bact. typhosum*. The cultures always contained Vi-antigen but were "O"-sensitive and her blood-serum had a Vi-titre of 1 : 10. Arrangements were then made to attempt to cure the carrier condition which, as far as one could ascertain, had existed for many years. Cholecystectomy was finally carried out and the gall-bladder, on being opened, was found to be of the strawberry type and to contain cholesterin calculi. The operation was carried out in a women's hospital at some distance from the present writer, and unfortunately the gall-bladder and its contents were not available for examination. However, after discharge from hospital the stools, in glycerine and saline, were forwarded at regular intervals and examination of a considerable series has failed to demonstrate the presence of *Bact. typhosum*. Vi-antibody, however, remained, and three months after the apparent focus had been removed the titre was unchanged at 1 : 10. It was not possible to undertake further examinations.

**Development of Vi-antibody in Inoculated Subjects during the Course of Diseases other than Typhoid Fever.**

Although a large amount of data on this subject has been collected, much of it was produced before the standardized Vi-serum had been taken into general use by all laboratories and it has been decided, for the purpose of

<table>
<thead>
<tr>
<th>Patient</th>
<th>Day of disease</th>
<th>Agglutinin Titre</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day of disease</td>
<td>TO</td>
</tr>
<tr>
<td>Pte. T</td>
<td>6</td>
<td>640</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>640</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>320</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>320</td>
</tr>
<tr>
<td>Pte. H</td>
<td>4</td>
<td>160</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>160</td>
</tr>
<tr>
<td>Miss I</td>
<td>?6</td>
<td>640</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>320</td>
</tr>
<tr>
<td>Pte. B</td>
<td>5</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>160</td>
</tr>
<tr>
<td>Boy N</td>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>Serjt. W</td>
<td>4</td>
<td>160</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>80</td>
</tr>
</tbody>
</table>
this communication, to rely on personal experience only, where the examinations were carried out with two standard antigens in parallel with the local standardized serum and controlled by the "provisional standard Vi-serum" of Felix. The number of individuals examined, excluding all possible cases of "Enteric Group," was only fifty and comprised cases of tonsillitis, bronchial catarrh, tuberculosis, and tropical typhus fever. These conditions were chosen because in inoculated subjects such diseases appear to cause, on occasions, non-specific stimulation of the "TO"-agglutinins.

The series included ten cases of tropical typhus and six of these patients showed the presence of Vi-antibody, and the agglutinin titres are set out in Table IV, p. 28.

It will be noted that there is a general tendency for the Vi-antibody to disappear as the agglutinins for Bact. proteus OXk develop. Definite Vi-antibody was not seen in any other condition investigated except doubtful enteric group cases.

The Production of Vi-antibody in Laboratory Animals following the Injection of Live Bacteria.

The possibility of the very early development of Vi-antibody in natural infections, in view of the appreciable Vi-titres observed occasionally within the first and second days of onset of typhoid fever, suggested the following experiments:

Experiment 1.—Five mice were given intraperitoneal injections of 20 million, a sub-lethal dose, of an eighteen-hour broth culture of Bact. typhosum Raw-Ben. The animals were killed at the intervals stated, blood removed by heart puncture, and the serum examined for Vi-antibodies.

<table>
<thead>
<tr>
<th>Animal killed after 24 hours</th>
<th>Vi-titre</th>
<th>Nil</th>
</tr>
</thead>
<tbody>
<tr>
<td>&quot; &quot; 48 &quot;</td>
<td>&quot; &quot;</td>
<td>Nil</td>
</tr>
<tr>
<td>&quot; &quot; 72 &quot;</td>
<td>&quot; &quot;</td>
<td>5</td>
</tr>
<tr>
<td>&quot; &quot; 96 &quot;</td>
<td>&quot; &quot;</td>
<td>20</td>
</tr>
<tr>
<td>&quot; &quot; 120 &quot;</td>
<td>&quot; &quot;</td>
<td>160</td>
</tr>
</tbody>
</table>

Bact. typhosum was recovered from the spleen cultures in all cases.

The above experiment was repeated upon fully grown white rats with double the dose of organisms. Daily "tail snippings" provided the serum which was pooled. Results of exactly the same order were obtained.

Experiment 2.—Six fully grown white rats were given 150 million of Bact. typhosum RV, grown in broth for eighteen hours, as intraperitoneal injections. The following day two of the animals were dead and the remaining four were extremely ill for the next thirty-six hours, after which they recovered quite quickly. Two of these rats were killed on the fifth day following the injection and their sera showed Vi-titres of 80 and 160 respectively and the other two had titres of 320 on the following day. As in the previous cases Bact. typhosum was recovered from the spleen cultures at the post-mortem examination.
Bacterium Typhosum

It is appreciated that the above experiments did not reproduce the conditions of a natural infection in man, for both rats and mice appear to recover very quickly after most severe illnesses and their antibody production seems to be less delayed than is the case of some larger animals. Nevertheless the results are suggestive.

The Production of Vi-antibody in Laboratory Animals following the Injection of Killed Vi-suspensions.

The majority of small laboratory animals respond readily to intravenous or intraperitoneal injections of Vi-suspensions. The response, however, to killed bacteria depends upon the sterilizing agent, the dose of organisms, number of injections, etc. The individual response in animals of the same species may also vary considerably.

Whilst it has been appreciated for some time that the presence of phenol in suspensions of Vi-strains is not conducive to the production of Vi-antibody, there are large numbers of sterilizing agents that do not appear to interfere with the agglutinogenic properties of Vi-suspensions although the production of other antibodies may be affected. The value of silver and mercury salts as such sterilizing agents has recently been demonstrated by Rainsford (1938, 1939) and in this laboratory mercury salts have been employed for several years for preserving and sterilizing "O"-suspensions, and for the last two years weak solutions of mercuric iodide have been used for killing and preserving Vi-suspensions for the preparation of Vi-agglutinating serum. Generally a large single dose of bacteria will produce on the fifth or sixth day, following the intravenous injection in a rabbit, a serum with a negligible "H"-titre and a relatively high Vi and "O"-titre. The difficulty with large doses for the inoculum is that they are very toxic and they have, in consequence, to be used with caution. According to Felix and Petrie (1938) formolized suspensions are unsatisfactory for the production of therapeutic sera, nevertheless the use of formalin as a detoxicating agent with large doses of concentrated suspensions for the preparation of Vi-agglutinating sera has given satisfactory results. The examples given below show the titres obtained during the preparation of such sera:

**Rabbit 43.**

11.5.39: "H"-, "O"-, and Vi-titres Nil. Given 750 millimetres formolized suspension T.R.V.  
16.5.39: "H"-titre nil. "O"-titre 1,000. Vi-titre 600.

**Rabbit 53.**

5.6.39: "H"-, "O"-, and Vi-titres nil. 500 millimetres T.R.V.  
10.6.39: Vi-titre 240. Rest rabbit ten days.  
20.6.39: 2,000 millimetres T.R.V.  
26.6.39: "H"-titre 10,000. "O"-titre 20,000. Vi-titre 4,000.
The Development of Vi-antibody following the Injection of Prophylactic T.A.B. Vaccine.

In most countries of the world the bacterial components of the T.A.B. vaccine are killed by heat and the suspensions preserved by the addition of phenol or an allied preparation. Felix and Pitt (1934) first drew attention to the deleterious effect of phenol on Vi-antigen, and this was confirmed by other workers. More recently Felix (1938) has suggested that the phenol does not destroy the antigen, but that its presence inactivates the agglutinogenic function of the Vi-substance. Even freshly phenolized suspensions of typhoid bacilli may fail to elicit any Vi response when injected into laboratory animals. Nevertheless when animals have suffered very severe reactions after the injection of large doses of such vaccines occasional Vi-titres have been noted on the fourth or fifth day following the inoculation. This and the following record led to the further investigation of this point:—

A healthy male developed a very severe reaction after receiving 0.5 c.c. of ordinary T.A.B. vaccine. The reaction commenced five hours after the injection with a rigor, and the pyrexia that followed lasted for four days. Fearing an attack of enteric fever blood was taken on the fourth day. The blood-culture was sterile but the serum had an "O"-titre of 40 and a Vi-titre of 80. On the twelfth day the Vi-titre had fallen to zero.

Twenty-five further individuals with similar histories have since been examined on the third or fourth day after inoculation. Nine of these showed a titre for Vi-antibody between 5 and 40 and in one case the agglutinins on the fourth day were as follows:—

| "H"   | 2,500 |
| "O"   | 250   |
| Vi    | 160   |

Experiment 3.—The twenty-four-hour growth of Bact. typhosum RV was washed off digest-agar slopes with 1 per cent phenol, well shaken, and then placed in the cold chamber, with occasional shakings, for seven days. The suspension was sterile after twenty-four hours, but further sterility tests were carried out on the fifth and sixth days. The strength of the suspension was then adjusted to contain 1,000 million organisms per c.c. in phenol-saline with a phenol concentration of 0.5 per cent. Ten fully grown white rats were given intraperitoneal injections of this suspension, each receiving 500 million organisms. The reactions were severe, but all the animals had recovered after forty-eight hours. The rats were divided into two batches and half were chloroformed on the fourth and half on the fifth day following the injection and bled out. The resulting sera were then examined for Vi- and "O"-antibodies. It is to be noted that the suspension injected was only faintly sensitive to a pure Vi-serum and failed to react with a pure "O"-serum. The following are the agglutinin titres of the sera examined:—
**Bacterium Typhosum**

<table>
<thead>
<tr>
<th>Rat number</th>
<th>Vi-titre</th>
<th>&quot;O&quot;-titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>80</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>40</td>
<td>160</td>
</tr>
<tr>
<td>3</td>
<td>160</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>60</td>
<td>20</td>
</tr>
<tr>
<td>5</td>
<td>80</td>
<td>80</td>
</tr>
</tbody>
</table>

**4th Day**

<table>
<thead>
<tr>
<th>Rat number</th>
<th>Vi-titre</th>
<th>&quot;O&quot;-titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>80</td>
<td>160</td>
</tr>
<tr>
<td>7</td>
<td>40</td>
<td>320</td>
</tr>
<tr>
<td>8</td>
<td>60</td>
<td>640</td>
</tr>
<tr>
<td>9</td>
<td>160</td>
<td>40</td>
</tr>
<tr>
<td>10</td>
<td>20</td>
<td>1,280</td>
</tr>
</tbody>
</table>

**5th Day**

<table>
<thead>
<tr>
<th>Rat number</th>
<th>Vi-titre</th>
<th>&quot;O&quot;-titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before injection</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>1st day</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>2nd</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>3rd</td>
<td>640</td>
<td>320</td>
</tr>
<tr>
<td>4th</td>
<td>320</td>
<td>320</td>
</tr>
<tr>
<td>5th</td>
<td>160</td>
<td>640</td>
</tr>
<tr>
<td>6th</td>
<td>80</td>
<td>1,280</td>
</tr>
</tbody>
</table>

Experiment 4.—A similar suspension of *Bact. typhosum* RV was prepared with 1 per cent formalin in place of the phenol. This suspension of 10,000 million was quite sterile in forty-eight hours' time. The formalin was diluted out with 1 : 10,000 mercuric iodide and the strength adjusted to 1,000 million per c.c. and 500 million given to seven rats as before. Pooled daily bleeds from tail-snippings were examined with the following results:

The strain RV is a Vi-culture which is deficient in "O"-antigen for, although the phenolized-suspension was only faintly sensitive to a pure Vi-serum, no "O"-agglutination could be obtained with either suspension. The formalin-mercury suspension was extremely sensitive to Vi-antibody.

The above two experiments do point to a very early production of Vi-antibody in rats and a fairly rapid fall in titre. In spite of the presence of phenol it would appear that special Vi-cultures can stimulate the production of Vi-antibody.

Experimental vaccines prepared after the manner described above have been given a limited trial in human subjects. The type of reaction observed with the standard T.A.B. vaccine was not recorded, but in place there was a definite heaviness of the arm and a general feeling of unfitness for three or four days. The trial was too small to be more than an indication, but the antigenic responses, "O" and Vi, were hardly superior to those noted with the ordinary phenolized vaccine. In view of the early fall that follows the rapid rise in Vi-titre in animals immunized with pure Vi-suspensions, and the successful mouse protection experiments with phenolized Vi-strains originally reported by Perry, Findlay and Bensted (1934), the advantage of the new form of vaccine does not seem proved. The agglutinin response of laboratory animals to the large doses employed for their immunization cannot be compared to the response of human subjects to ordinary vaccines.
Discussion.

The presence of Vi-antigen in recently isolated cultures of Bact. typhosum is now too well recognized to require comment. In very few of such cultures does there appear to be a complete absence of this antigen, and in the majority it seems to be well developed, although there are not large numbers, when put to the severe test, that fail to show any reaction with a pure "O"-serum unless they are grown on special media. The slow growth on egg-medium in a reduced oxygen tension, as suggested by Gladstone (1937), would appear to enhance Vi production; as a result of cultivation by this technique numbers of "O"-sensitive strains have become completely "O"-resistant and once in this stage the strains, with ordinary care, are remarkably stable. Occasionally, however, a strain may revert for some unknown reason. Although it is a rare happening it is advisable to examine all stock cultures of Vi-strains from time to time from this point of view.

The fact that Vi-antigen can be encouraged to develop in the classical "T.901.O"-strain under the conditions described is not considered important, for there is no difficulty in retaining this strain in its original form as a pure "O"-culture.

The production of the Vi-forms deficient in "O"-antigen is of considerable practical value for, since it has been shown that such strains can be shaken with alcohol to remove the reacting part of the flagella, the production of pure Vi-antigens (for the estimation of Vi-antibody) is not limited to such strains as "T.Vi.1." Indeed there is some evidence, not yet sufficient to be definite, to suggest that these artificially produced pure Vi-suspensions are superior in their specificity.

Although these Vi-suspensions show no agglutination with pure "TH" or "TO" serum, yet they are capable of producing satisfactory "H" and "O" titres when injected into animals so that even in the dead bacteria there must be some efficient masking of these antigens. When the Vi-sensitivity has been much reduced by prolonged contact with alcohol or phenol such suspensions still fail to react with "O"-antibody.

The occasional failure to cultivate Bact. typhosum from the blood of patients in the early stages of the disease has not been stressed in this communication, for the importance of blood culture in the diagnosis of typhoid fever is not under discussion. Undoubtedly there are occasions when the technique or the media are at fault, but it is equally true that at other times the same technique and the same batch of media will give successful results four or five days—sometimes even two weeks—after a series of failures.

Whatever may be the true story of the initial pathology of typhoid fever it is generally thought that, before the bacteræmia that signifies the clinical establishment of the disease, there is multiplication of bacteria in other tissues. It is, therefore, possible that on occasions the reaction in those tissues or cells may be great enough to become a clinical entity and a local barrier immunity be developed to prevent a bacteræmia. The barrier would eventually break down and bacteria finally swarm into the blood-stream.
It is suggested that tissue reactions of this sort may be concerned in the early development of Vi-antibody; in the only case of this nature that has been fully investigated the blood-serum in the first few days of the disease had an appreciable Vi-titre but no "O"-agglutinins. Repeated blood cultures in the early stages were negative although the organism was recovered with ease later. The estimation of Vi-antibody calls for considerable care, experience, and critical ability, but the technique itself is not complicated, although great attention must be paid to detail. Unless a serum is really clear and free from hemoglobin no attempt should be made to carry out the test.

It is disappointing that the development of Vi-antibody cannot be demonstrated in every case of typhoid fever. The agglutinin curves of many of the cases under review were of less help than the estimation of "O"-antibody. The value of any such test depends upon its practical application in the diagnosis of disease. It is not suggested that Vi-antibody is not produced in every case of a typhoid infection, but it is maintained that its detection is frequently not a practical proposition for the routine clinical laboratory. The demonstration of Vi-antibody in the serum during the course of a continuous fever may be highly suggestive, but it is felt that considerable caution should be exercised before accepting the presence of this antibody as specific evidence of a typhoid infection or its absence as excluding such a condition.

It is of interest to note that Vi-antibody may develop during the early stages of tropical typhus fever, but it will be observed that there was no question of confusion concerning the diagnosis; in most cases the Vi-titres were falling before the OXk-titres had reached very high figures. It is not clear whether the production of Vi-agglutinins is stimulated in any other infective condition. Positive evidence on this point has not been observed by the present writer, but the series of cases is as yet too small to form any definite opinion.

For some time evidence has been accumulating that the chronic typhoid carrier regularly shows the presence of Vi-agglutinins in the blood serum (Felix, Krikorian and Reitler, 1935; Giovanardi, 1936, 1937; Pijper and Crocker, 1937; Felix, 1938; Bhatnagar, 1938). Out of the seven reported in this communication definite amounts of Vi-antibody were demonstrated in six of the carriers. It is possible that the seventh individual was an intermittent carrier, but although traces of Vi-antibody were found in dilutions of 2\(^{1/2}\) of the serum all higher dilutions from 1 : 5 upwards were negative. The fact that the "carrier" that has apparently been cured of her condition still retains her Vi-antibody is hardly surprising; there are other examples of immune bodies, developed during chronic infections, remaining long after a cure has been effected.

The higher Vi-titres found in animals following the injection of either live or dead bacteria, than those encountered in human subjects, is perhaps more marked than is the case with other antibodies. The very early response recorded in experimental infections would appear to have its parallel in some
natural infections in man where appreciable titres have been found immediately the disease has established itself, and it is reasonable to suppose that the antibody was developing during the incubation period.

It was suggested by Bhatnagar (1938) that inoculated subjects developed Vi-antibody more quickly during an infection than the uninoculated. The demonstration of transitory Vi-titres in certain individuals following ordinary phenolized T.A.B. vaccine, and the similar early response to large doses of phenolized suspensions of Vi-strains in rats and other immunity reactions in connexion with this antigen, suggest that in most cases the measurable response is shortlived but that residual antibodies in minimal amounts may persist to play their part in the reaction that follows an infection. The true explanation of the part that the Vi-antigen plays in infection and resistance, however, is still awaited.

Summary.

(1) An account is given of the Vi-content in recently isolated strains of Bact. typhosum, the maintenance of this antigen, and its development in cultures grown on egg-medium under reduced oxygen tension.

(2) The production of pure Vi-suspensions from strains other than "T.Vi.1" is described.

(3) A record of Vi-estimations in 80 cases of typhoid fever is given. At the very least, five cases failed to show any development during the whole of the illness from the third day onward.

(4) Vi-antibody was demonstrated in six out of seven chronic carriers and in two out of four convalescent carriers.

(5) The question of non-specific stimulation of Vi-antibodies is discussed.

(6) Certain experiments in relation to the animal response to the injection of live and dead bacteria are described, and the transient Vi-titres that were obtained are noted.

References.


