AN INQUIRY INTO THE NATURE OF THE SEROLOGICAL DIFFERENCES EXHIBITED BY DIFFERENT CULTURES OF A BACTERIAL SPECIES (*B. TYPHOSUS*)[1].

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(With 5 Charts.)

A Report to the Medical Research Council.

In a previous communication[2] one of us showed that certain strains of *B. typhosus* presented marked serological differences. The experiments, to which we now propose to make further reference, were conducted in the following manner.

Four strains were taken, and a quantity of agglutinable culture of each strain was prepared and standardised for opacity. These cultures were then used in making parallel daily tests of the serum of a rabbit, successively immunised against three of the strains by means of single intravenous inoculations of their standardised cultures. The general character of the results was in agreement with conclusions reached by one of us in previous work (1899–1901)[3], but showed still greater serological differences between the strains. Thus, the evidence obtained separated the four strains into two serological groups, two strains falling into each group.

The three successive inoculations (on days 1, 8 and 16) were made with three different strains, and after each inoculation the titre of the serum rose for the homologous group to a point very greatly higher than its titre for the strains in the heterologous group. These observations have already been figured for the first 14 days of the experiment (*loc. cit.*). But the whole data obtained during 25 days are now recorded in standard agglutination units (Table I), and the readings for days 7, 13, 18 and 25, which illustrate the points of special interest, are charted in the accompanying diagram (Chart 1).

The cultures used are indicated as T.E., T.L., T.O., and T.T. The rabbit was inoculated intravenously on day 1 with 0·1 c.c. (25 million approximately) of T.L., on day 8 with an equal dose of T.T., and on day 16 with the same dose of T.O. The tests of the serum were put up by one of us, and were read by the other (A. D. G.) who was kept in ignorance of the details of the test before him until the readings had been made and recorded.

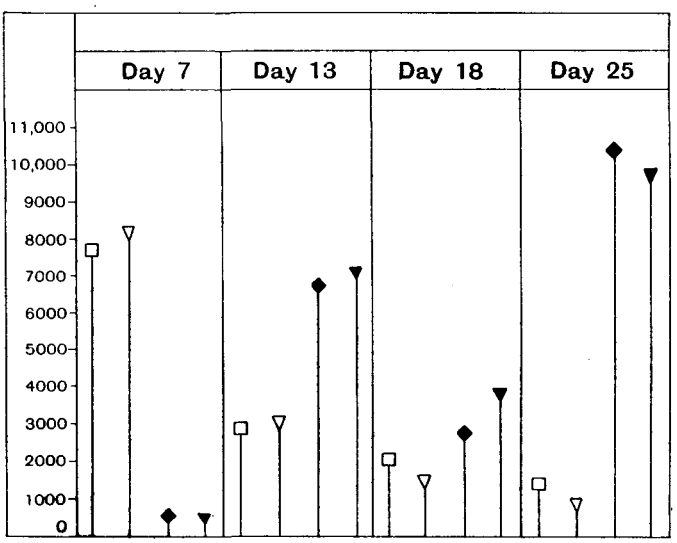
[1] Received February 17, 1921.

[2] Walker, E. W. Ainley (1918). *Journ. of Hygiene*, xvii. 380.

[3] Walker, E. W. Ainley (1901). *Journ. of Path. and Bact.* vii. 250.

Table I.

Day	Culture T.E.	T.L.	T.O.	T.T.
1	<14	<14	<14	<14
2	—	—	—	—
3	—	—	—	—
4	65	75	17	14
5	1925	1425	300	345
6	6900	6450	600	570
7	7800	8300	730	600
8	6800	7800	780	570
9	6450	6900	565	513
10	—	—	—	—
11	3450	3850	1090	1290
12	3225	3150	2538	2463
13	3038	3225	6900	7300
14	2960	2920	5600	5000
15	2580	2780	4920	4150
16	2000	1460	2900	3000
17	—	—	—	—
18	2260	1660	2900	4000
19	2000	1360	5540	5000
20	1360	1300	6230	4550
21	1965	1170	9750	9300
22	1695	915	10350	10200
23	—	—	—	—
24	—	—	—	—
25	1650	1000	10580	9950



"Response" = highest titre observed.

Chart 1.

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It is clear from the tabulated results of the experiment just described that T.E. and T.L. may be regarded as constituting one group (Group 1), and T.O. and T.T. as constituting another group (Group 2), which present definite and well-marked serological differences. That fact was sufficient for the purpose in view at the time confirming, as it did, and amplifying the old experiments already referred to, which were also adduced in evidence. No further explanation of the phenomenon was suggested, and its discussion was purposely withheld, partly because it might have distracted attention from the clear and simple issue under examination, but still more because it would have trenched upon the province of another communication dealing with the question of velocity of reaction in relation to agglutination, which one of us (A. D. G.) was then intending shortly to make. Circumstances subsequently prevented that intention from being carried out, and misunderstandings in regard to the interpretation of these experimental results, and doubts as to their accuracy, have since arisen.

On the one hand it has been stated that the phenomenon has never been observed by other workers, and does not occur. On the other hand the observations have been taken to supply an argument against reliance on the technique and methods of interpretation of agglutination tests introduced and developed by Dreyer.

In evidence that the phenomenon already twice described by one of us[1] does actually occur, we may state at once that in the course of the experiments to be described below we have recorded the following observations:

1. We have reproduced the phenomenon in question twice over with the identical material of cultures T.E. and T.O. (of which a supply fortunately remained over in our possession), using two fresh rabbits. One of these was first inoculated with T.E. and subsequently with T.O., while the other was first inoculated with T.O. and then with T.E.

2. We have prepared two other cultures of *B. typhosus* which exhibit similar serological differences, and have demonstrated this difference ten times in experiments on four other rabbits.

3. Finally we have found that the sera prepared by the inoculation of one member, or both members in succession, of either of these pairs of cultures exhibit the same serological difference with the other pair as they exhibit with their own pair of cultures.

The reliability of the observations being thus placed beyond reasonable doubt, the question next arises whether this phenomenon and its interpretation should diminish or increase our confidence in the reliability of Dreyer's method of performing agglutination tests and interpreting their results. On this point we will only offer one comment at present, namely that we are quite unable to see how, by any other method known to us, the occurrence of

[1] Walker, E. W. Ainley (1918). *Journ. of Hygiene*, XVII. 380; Walker, E. W. Ainley (1901). *Journ. of Path. and Bact.* VII. 250.

the phenomenon could have been proved so indubitably, and the observation freed from problematical and purely speculative hypotheses.

In the communication already mentioned as being questioned and misunderstood the following statement was made: "It is not any part of my present intention to discuss the meaning of the very striking differences in agglutinability exhibited by these four strains of *B. typhosus* under the experimental conditions just described. They are associated with other interesting and well-marked characteristics which do not bear immediately upon the question at issue." These other characters will shortly be referred to, but before this is done it appears to be essential to reach a clear understanding as to what is meant by the term "strain," since we have been informed that its use has been misunderstood. In making use of this term we ourselves do not assume at the outset anything in reference to the cultures so spoken of, except that they have been obtained from different sources, derived by different methods of cultivation, or selected for any purpose from a single culture. This we believe is in agreement with ordinary usage. We do *not* assume that they are necessarily in any way different from each other, and still less that they present any *permanent* differences. Whether differences in respect of particular characters exist or not, is always a matter for investigation in each particular case.

That permanent differences exist between different examples of particular well-recognised pathogenetic bacteria we are always inclined to disbelieve until strong evidence to the contrary is forthcoming, since the experience gained in the study of certain forms (streptococcus, meningococcus) seems to show that there is sometimes a tendency for authors to assume permanence and specific value for differences which turn out to be shifting in character, or even interchangeable. But where such differences are proved to exist the forms concerned will constitute species or varieties, and will not be spoken of as strains.

Of the typhoid strains T.E., T.L., T.O. and T.T., the experiment already quoted shows that the first two, T.E. and T.L., were not greatly different from each other serologically; but they differed serologically from T.O. and T.T., which again were serologically similar to each other. Nevertheless, one of us (A. D. G.) was able to obtain quite easily from the strain T.O.—the only one then tried—subcultures which were serologically similar to T.E. and T.L.

It is a common observation that the cultures of any given bacterium obtained from different sources may differ to a greater or less extent in some of their characters. The individuals which compose the population—as we may call it—of a bacterial culture are not identical, but differ among themselves. So do the progeny of any single bacterium. Accordingly, whether that bacterium is propagated in the ordinary way, or through individual colonies by a succession of platings, its variable characters will sooner or later exhibit their variations in the course of repeated subculture. Still more may this be seen if the micro-organism is subjected to more or less diverse environmental

conditions. And this has inevitably been the case with strains obtained from different sources.

Since the population of a bacterial culture is composed of individuals which differ among themselves it is quite possible to demonstrate indications of diversity among the primary subcultures of a series of colonies obtained by plating out from a single colony. And it would theoretically be possible to determine the limits of variation exhibited by the progeny of a single bacterium by examining them all in the primary subcultures of all the colonies on a series of plates. But whether the variations exhibited by strains and variants obtained either from different sources or by intentionally subjecting the micro-organism to an altered environment are greater than the differences existing among the population of a single colony cannot at present be conclusively determined. Yet it is of interest that in the experiments recorded below we found that two colonies selected from a particular plating of a population of *B. typhosus* yielded on subculture in bouillon two cultures, T.M., and T.Non. differing as widely serologically as any two of the strains chosen from different sources. This result is in complete and confirmatory agreement with the statement repeatedly made to us by Professor Dreyer, that he has never in his own experience seen greater differences between so-called strains of these organisms than may be found in different cultures of the same strain.

Description of Experiments: First Series.

The characters which distinguished the strains T.O. and T.T. from T.L. and T.E. were non-motility and relative inagglutinability with ordinary standard typhoid agglutinating serum. The association of relative inagglutinability with non-motility was, in our own work, first noted by A. D. G., but we subsequently became acquainted with the fact that it had been observed by Malvoz and studied by Nicolle and Trenel, and later by Theobald Smith. To their investigations and conclusions we shall have occasion to return.

The experiments now to be recorded were conducted in the following manner. Four rabbits (B 1, B 2, B 3, and B 4) were used in the preparation of serums for investigation of the cultures T.M. and T.Non. The latter were formolised bouillon cultures, each derived from a single colony picked off the same plating of one of his stock *B. typhosus* cultures (T.E.) by A. D. G. T.Non. was from a colony which, when grown for 24 hours in bouillon, gave rise to a population apparently devoid of motile elements. A similar population derived from the T.M. colony, consisted almost entirely of actively motile bacilli. The cultures were diluted with formolised normal saline solution to standard opacity precisely as in the preparation of standardised agglutinable cultures of *B. typhosus*, and the whole of the experiments were carried out with one and the same stock suspension of each type of bacillus. Two other rabbits (B 5 and B 6) were used in preparing serums for the re-examination of the cultures T.E. and T.O., of which a quantity remained over in cold storage from the experiments of February and March 1918 recorded above.

The rabbits were immunised by intravenous inoculations of the formolised cultures, each inoculation consisting of 1 c.c. of the culture concerned, containing approximately 250–300 million bacilli. Thereafter the animals were bled (from the ear) for the preparation of serum on the eighth day, counting the day of inoculation as the first day.

Somewhere about 30 c.c. of blood were taken at a bleeding, and the serum was preserved by the addition of 0·3 per cent. of phenol. After each bleeding a period was allowed to elapse before the next inoculation; and before such inoculation was made a sample of blood (3 or 4 c.c.) was collected, and serum prepared, in order to determine by agglutination tests the starting point of the reaction to the second or third dose of antigen.

The rabbits used were all over 2000 grms. in weight, with an average of 2490 grms., and they remained in good condition throughout the experiments. The scheme of the inoculations is shown in Tables II and III.

Table II.

Rabbit	1st inoculation day 1 antigen	2nd inoculation day 15 antigen	3rd inoculation day 42 antigen
B 1	T.M.	T.M.	T.Non.
B 2	T.M.	T.Non.	0
B 3	T.Non.	T.M.	0
B 4	T.Non.	T.Non.	T.M.

T.M. = motile form, T.Non. = non-motile form, isolated from a population of T.E.

Table III.

Rabbit	1st inoculation day 1 antigen	2nd inoculation day 19 antigen
B 5	T.E.	T.O.
B 6	T.O.	T.E.

T.E. = motile strain, T.O. = non-motile strain.

It will be seen that each rabbit was immunised first with one dose (B 2, B 3, B 5, B 6), or with two successive doses (B 1, B 4) of one of a pair of typhoid cultures, and that subsequently it received a single dose of the other member of the same pair of cultures. And each pair of cultures consisted of a motile and a non-motile form of micro-organism.

The bleedings made one week after each inoculation, and the small sample of blood taken immediately before giving a second or third inoculation, provided us with a total of 22 serums. These serums were tested out in the usual way with Dreyer's technique against their own pair of cultures. Furthermore the serums of rabbits B 5 and B 6 (prepared with T.E. and T.O.) were tested also against cultures T.M. and T.Non.; and a chosen four of the serums of the first series of rabbits prepared with T.M. and T.Non. were tested against T.E. and T.O. as well as against their own cultures.

The results obtained are given in Table IV as end-point readings, after the tubes had remained in a water bath at 51–53° C. for two hours, and subse-

quently at room temperature for 24 hours. The figures in the table represent the highest dilution in which agglutination could be detected naked-eye after the lapse of this period. Preliminary readings were also taken in the usual way at the end of the period of incubation in the water bath. But, owing to the slow rate of flocculation of the non-motile cultures, the 24-hour readings seemed to give a fairer and more satisfactory basis of comparison, and are therefore alone made use of here. The series of dilutions employed was 1 in 25, 50, 125, 250, 500, 1000, 2500, 5000, 10,000, 20,000, 50,000 and 100,000.

Table IV.

Rabbit	Serum	Antigen used	Readings with agglutinable culture			
			T.M.	T.Non.	T.E.	T.O.
B 1	After 1st inoculation	T.M.	5,000	2,500	—	—
	Before 2nd ,,	—	12,500	4,000	—	—
	After 2nd ,,	T.M.	20,000	5,000	20,000	5,000
	Before 3rd ,,	—	10,000	1,000	—	—
	After 3rd ,,	T.Non.	7,000	2,500	—	—
B 2	After 1st ,,	T.M.	4,000	1,000	—	—
	Before 2nd ,,	—	5,000	1,000	—	—
	After 2nd ,,	T.Non.	5,000	2,500	—	—
B 3	After 1st ,,	T.Non.	500	5,000	500	5,000
	Before 2nd ,,	—	500	2,500	—	—
	After 2nd ,,	T.M.	10,000	2,500	20,000	10,000
B 4	After 1st ,,	T.Non.	125	2,500	—	—
	Before 2nd ,,	—	300	2,500	—	—
	After 2nd ,,	T.Non.	700	5,000	1,000	20,000
	Before 3rd ,,	—	500	2,500	—	—
	After 3rd ,,	T.M.	10,000	5,000	—	—
B 5	After 1st ,,	T.E.	2,500	1,000	2,500	1,000
	Before 2nd ,,	—	2,500	500	2,500	500
	After 2nd ,,	T.O.	2,500	10,000	2,500	10,000
B 6	After 1st ,,	T.O.	50	2,500	50	2,500
	Before 2nd ,,	—	25	1,000	50	1,000
	After 2nd ,,	T.E.	10,000	10,000	10,000	10,000

NOTE: A dash in a column of readings means that the serum in question was not tested on the culture concerned. There was not enough material remaining of T.E. and T.O. to carry out the whole series of determinations

EXPERIMENTAL RESULTS.

In reading the table it will be noted that the end-point readings of T.E. and T.O. often show an absolute coincidence with those of T.M. and T.Non. respectively. The coincidences would doubtless have been less absolute had a more finely graded series of dilutions been employed. For instance if two cultures both give some kind of "trace" at 1 in 5000 with a particular serum, and nil at 1 in 10,000, it is possible that the actual end-point of the one might be at 1 in 6000, and of the other at 1 in 8000. But the pursuit of finer distinctions was not material to the present investigation.

The results are exhibited graphically in Charts 2, 3 and 4. An examination of these charts and of Table IV brings out the following points:

1. Both "motile" (T.M. and T.E.) and "non-motile" (T.Non. and T.O.) types of *B. typhosus* give rise on inoculation, in all the animals used, to the production of serums which agglutinate to a greater or less degree both types of bacillus.

2. T.M. and T.Non. present a marked "serological difference." In each of the four rabbits used the serum obtained after a single inoculation, or after two inoculations of the same antigen, acts much more strongly on the homologous bacillus than on the heterologous type. The difference ranges from double up to as much as twenty-fold.

3. T.E. and T.O. show a similar "serological difference," the range in this case running to fifty-fold.

4. T.M. and T.E. exhibit a completely homologous serological character, and the same is true of T.Non. and T.O. For wherever T.M. is the more highly agglutinated of its pair, T.E. presents a similar relation to T.O., and the converse holds equally good. But T.M. and T.E. run together, as also do T.Non. and T.O.

5. In every case where a non-motile type of bacillus was used as antigen in the production of a given serum, the "serological difference" between the types is distinctly greater than where a "motile" antigen was employed.

6. When, after one (or two) inoculations with one type of bacillus (motile or non-motile as the case may be), a rabbit is inoculated with the other type of bacillus, its serum exhibits striking changes in relative agglutinating power. The titre always shows a notable increase for the antigen last injected. For the other antigen the naturally occurring fall frequently continues, though it may be arrested or even slightly reversed. But in any case the ratio of the titres is always completely changed, and this reversal or "cross-over" is the most striking feature of the charts.

We are of opinion that the phenomena just described must be taken into consideration in all observations of serological differences before these are interpreted as affording satisfactory proof of the existence of permanent varieties within a species of bacteria.

The Agglutinative Characteristics of the two Types.

Not only do these two types of *B. typhosus* exhibit the agglutinogenic differences just described, but they also present quite definite and constant differences in their manner and rate of clumping. The motile type forms large, fluffy flocculi in the stronger dilutions, and shows rapidly descending gradation in the size of the clumps as the end-point is approached. The reaction is in general nearly complete at the end of two hours in the water bath.

The non-motile type on the other hand forms small, compact and granular clumps, very similar to those formed by dysentery bacilli. There may be no reading higher than "trace" at the end of two hours incubation in a series of

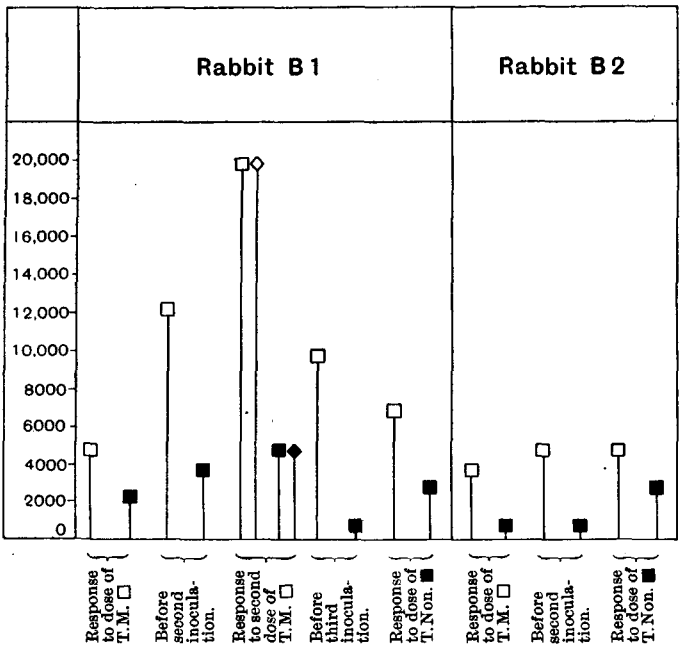

Chart 2.

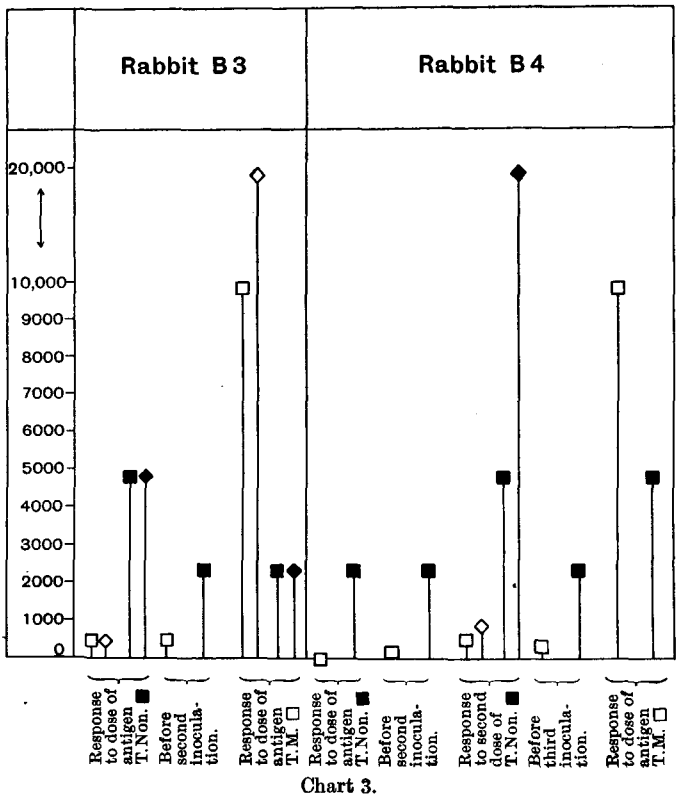

Chart 3.

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six or seven dilutions, all showing agglutination, though, after 24 hours, readings approaching "total" appear in the stronger dilutions from the coalescence and sedimentation of the small clumps.

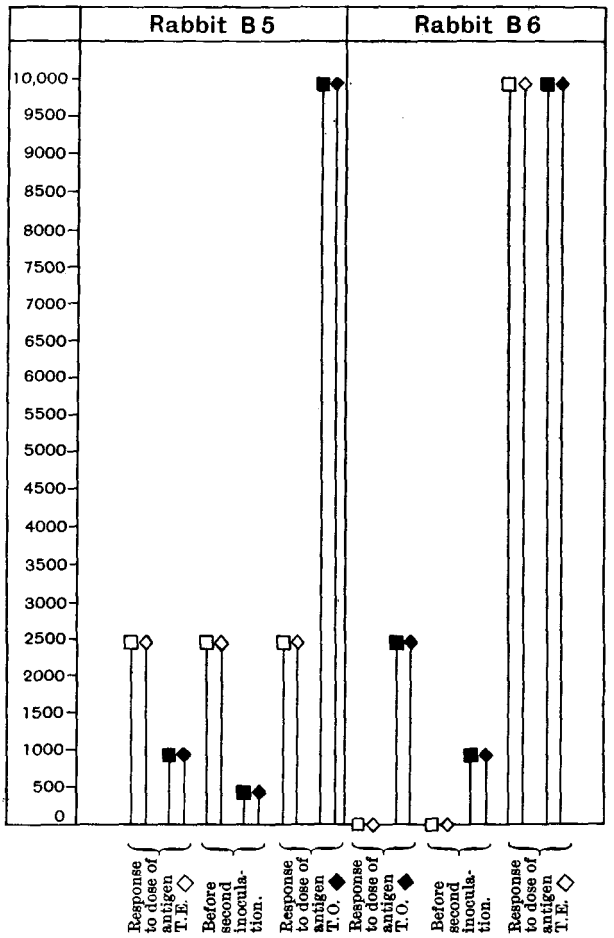

Chart 4.

DESCRIPTION OF EXPERIMENTS AND RESULTS: SECOND SERIES.

When the experiments described above had been completed, and the results worked out, it was suggested to us by Professor Dreyer that some further light might be thrown on the nature of the phenomena observed by conducting a series of experiments with *washed* bacilli. Accordingly a portion of each of the suspensions T.M. and T.Non. was centrifugalised to deposit its bacteria, and the clear supernatant fluid was removed[1]. The bacteria thus

[1] In agreement with the fact that T.Non. deposited its bacilli on standing much more quickly (in two or three days) than T.M., it required much less centrifugalisation than the latter to obtain a complete separation of its bacilli.

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separated were washed four times with successive quantities of normal saline solution in the usual manner, and were finally suspended in a volume of 0·1 per cent. formolised normal saline solution equal to the original volume of the bacterial suspension.

These suspensions of washed bacilli were used in immunising two pairs of rabbits (B 7 and 8, and B 9 and 10). B 7 and 8 were treated with T.M. and B 9 and 10 with T.Non. Each rabbit received intravenously an inoculation of 1 c.c of the appropriate bacterial suspension on day 1 and day 9 of the experiment, and was bled for the preparation of serum on day 16. The serums were tested out against T.M., T.Non., T.E., and T.O. The readings obtained are given in Table V, and charted in Chart 5.

Table V.

Rabbit	Inoculation	Culture agglutinated	"Titre"	Culture agglutinated	"Titre"
B 7	Washed	T.M.	17000	T.E.	18000
	T.Motile	T.Non.	2500	T.O.	5000
B 8	Washed	T.M.	3500	T.E.	2500
	T.Motile	T.Non.	2500	T.O.	6000
B 9	Washed	T.M.	50*	T.E.	50*
	T.Non-motile	T.Non.	1000	T.O.	3500
B 10	Washed	T.M.	25*	T.E.	<25*
	T.Non-motile	T.Non.	1000	T.O.	3500

* For significance of these figures see text (Discussion).

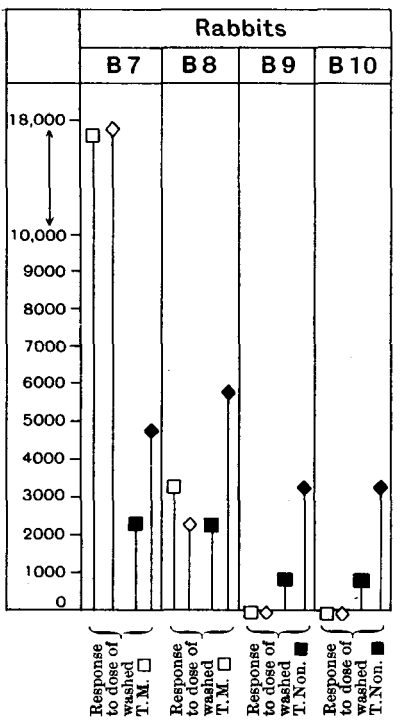

Chart 5.

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It will be seen that the washed bacilli gave results, which, though in the main similar to those already described, yet differ in certain important points.

1. In both rabbits treated with the non-motile form the serum produced had so extremely feeble an action on the motile forms that one cannot be certain that the readings exceed the range of possible "normal" agglutination.

This does not, however, constitute an absolute distinction between washed and unwashed bacilli, since in one rabbit (B 6) of three treated with unwashed organisms, the readings were as low as these. Yet the other two rabbits of that series gave quite good agglutination of the motile form. Hence it seems that the difference must be dependent upon the idiosyncrasies of individual rabbits, and not upon differences inherent in the antigens.

2. In one of the two rabbits (B 8), injected with a suspension of the washed motile form, the serum showed relatively high agglutination of the corresponding non-motile suspension (T.Non.), and actually agglutinated the other non-motile suspension (T.O.) in a much higher dilution than either of the motile suspensions (T.M. and T.E.). But the other rabbit (B 7), injected with the same suspension, gave a serum whose action was precisely similar to that of the serums produced by the injection of suspensions of *unwashed* motile forms in the first series of experiments.

DISCUSSION.

The association of inagglutinability with non-motility appears to have been noticed first in *B. typhosus* by Malvoz[1] in 1897. He believed that his experimental treatment of the bacilli removed their flagellated envelope, and that both phenomena were the result of the loss of these structures.

Nicolle and Trenel[2], 1902, studied variations of agglutinability and of agglutinogenic power of *B. typhosus* and certain other organisms. From one of these which they described as "similar to *B. typhosus*," they produced experimentally motile and non-motile strains, prepared serums with both, and carried out cross-agglutination tests. The "motile" serum agglutinated the motile, but not the non-motile form; whereas the "non-motile" serum agglutinated neither. These results with the non-motile form of the organism stand in contrast with the observations recorded above, and we are inclined to think that a probable explanation of the discrepancy lies in the lack of delicacy of the technique in use at that date. No detailed description of their methods is given by these authors; but the technique which seems to have been in use among French workers at that period was not likely to reveal the minute and very slowly forming clumps of non-motile forms. For example, one or two hours at room temperature would fail to produce any visible clumping.

[1] Malvoz, E. (1897). *Annales de l'Inst. Pasteur*, XI. 582.

[2] Nicolle, C. and Trenel, M. (1902). *Annales de l'Inst. Pasteur*, XVI. 562.

To reach reliable conclusions concerning the presence or absence of agglutination a technique is required which includes the following features:

1. Heating at the optimum temperature for an adequate period.
2. A further more extended period at room temperature for the completion of the reaction. This is especially important in the case of organisms which do not readily form easily visible clumps.
3. The use of artificial light and a dark background for taking readings; since fine clumping cannot be read properly in any other way.

These requirements are best met, so far as we are aware, by using Dreyer's methods.

Furthermore it is important, so far as possible, to carry out investigations of this character with the same batches of bacterial suspensions throughout the whole series of experiments. For this procedure automatically excludes all question of the relative agglutinability of different batches.

Theobald Smith and Reagh[1], who worked with motile and non-motile forms of "Hog-cholera" bacillus, came to the conclusion that there existed two kinds of agglutinin, the one acting on flagella, the other only on the bacterial bodies. The non-motile form gave rise in animals only to the production of the "body agglutinin," whereas the motile form produced agglutinins for both flagella and bodies. To demonstrate the "body agglutinins" a much higher degree of immunisation was necessary than for the production of "flagella agglutinins."

In our own experiments no higher degree of immunisation was required for the production of agglutinins by the non-motile form. Precisely the same immunising doses were employed in both cases. The non-motile suspension, when unwashed, produced in two animals out of three serums which possessed agglutinating action on the motile form as well as on itself, though their action on the former was relatively low. But both serums, made with the same bacilli after washing, failed to give conclusive evidence of agglutinin production for the motile form.

Benians[2] obtained an inagglutinable form of dysentery Shiga from a chronic abscess in a guinea-pig, which had been inoculated with "agglutinable" Shiga culture. He found that the inagglutinable form produced no agglutinins for either form. It also failed to absorb agglutinins from an ordinary Shiga serum. But his experimental data are too scanty to justify a final conclusion on these points.

In a short note, Arkwright[3] describes the derivation of two forms S. and R. from a number of strains of dysentery, typhoid and several other organisms. The S. form of dysentery Shiga agglutinates in large clumps, the R. form only in fine granules. Both forms agglutinate to the same titre with stock Shiga serum. But when serums are prepared with the S. and R. forms respectively

[1] Smith, Theobald and Reagh, A. L. (1903–4). *Journ. of Med. Research*, x. 89.
[2] Benians, T. H. C. (1920). *Journ. of Path. and Bact.* xxiii. 171.
[3] Arkwright, J. A. (1920). *Journ. of Path. and Bact.* xxiii. Proceedings, 358.

only the homologous culture is agglutinated "in the higher dilutions," and "very little cross-agglutination took place." The data actually given show an appreciable amount of cross-agglutination, in the case of S. serum, and with R. serum show a certain degree, which might have appeared more evident had any test been made below a dilution of 1 in 80.

The author states that somewhat similar results were obtained with *B. typhosus* and with *B. dysenteriae* (Flexner-Y)[1].

The subject of the existence of two different agglutinogenic forms of certain bacteria has also been studied recently by Feiler[2] in the case of *B. typhosus* and *B. paratyphosus* B.; by Weil and Felix[3] for *Proteus* x. 19, and also for certain members of the paratyphoid-enteritis group[4]; by Börnstein[5] and by Bach[6] for *Proteus* x. 19 and by Breinl[7].

The majority of the authors referred to support the view that in these cases two different antigenic factors are in question, which lead to the production of two different agglutinins, but whereas the earlier workers, who investigated motile species only, attributed the differences to the presence or absence of flagella; the discovery by more recent observers of similar phenomena in non-motile species would seem to show that this view is inadequate. Since the two forms are found in motile and non-motile species alike it seems probable that the presence or absence of flagella does not provide the whole explanation, even in the case of motile bacteria.

Our own experiments are not inconsistent with the hypothesis of certain authors that two different agglutinogens exist in variable proportions in the bacterium. On the other hand they do not fully accord with the theory of "body agglutinins" and "flagella agglutinins" as formulated by Theobald Smith and Reagh, since the agglutinins produced in our experiments by the non-motile form, give the same kind of large, fluffy flocculi in suspensions of the motile form as are produced by the homologous serum, though the titre is lower. This fact stands in contrast with the statement of Smith and Reagh that "body agglutinins" are only capable of producing in either form a fine granular clumping.

The evidence at present available does not seem to us to suffice for the formulation of a convincing hypothesis. But if the theory that these bacilli possess two different agglutinogenic properties or factors be accepted provisionally, the following conclusions will follow from our experiments:

1. Every culture of *B. typhosus* examined contains both antigenic factors.
2. The temporary preponderance of one or other agglutinogenic property

[1] Since this Article was sent in to the Medical Research Council for publication Arkwright's full paper has appeared: (1921). *Journ. of Path. and Bact.* XXIV. 36.

[2] Feiler, M. (1920). *Ztschr. f. Immunitätsf. u. exper. Therapie*, Orig. XXIX. 303.

[3] Weil, and Felix, A. (1920). *Wien klin. Wochenschr.* XXX. 1509.

[4] Weil, E. and Felix, A. (1920). *Ztschr. f. Immunitätsf. u. exper. Therapie*, Orig. XXIX. 24.

[5] Börnstein, P. (1920). *Ztschr. f. Hyg. u. Infekt.* XC. 206.

[6] Bach, F. W. (1920). *Centralbl. f. Bakt.* Abt. I Orig. LXXXIV. 265.

[7] Breinl, F. (1920). *Ztschr. f. Immunitätsf. u. exper. Therapie*, Orig. XXIX. 49.

in the mass of the population of a particular culture determines the serological character of that culture.

3. But the character is only temporary, for it is possible to select *from a single culture* individuals which will produce populations differing as widely serologically as any two "strains" obtained from different sources, or derived by different methods of cultivation. Moreover, a non-motile culture will, under suitable conditions, always (in our experience) yield eventually a fully motile growth.

4. That these different factors both reside within the bacteria themselves, and that the fine granular clumping of the non-motile culture is *not a precipitin reaction* between serum and culture fluid, are demonstrated by our experiments with the washed bacilli.

5. It is clear that in the motile form, whether unwashed or washed, both properties are always present. They are also present in the unwashed non-motile culture, but conclusive evidence of the presence of the "motile" antigenic factor in the washed non-motile suspension is lacking in our experiments. Yet, since this form will readily give rise to the motile form on subculture, we adhere to the view that each individual bacillus, whether in the motile or non-motile phase, possesses both potentialities.

Certain questions affecting the significance and interpretation of these phenomena are under further investigation. Meanwhile the bearing of these observations on the practice of standardising agglutinable cultures must now be considered briefly. It is clear that for the preparation of such cultures it is necessary to use the readily agglutinable form of the bacillus. And in Dreyer's *Directions for the Preparation of Standard Agglutinable Cultures*, it is laid down that repeated subculture in broth should be carried out, for the purpose of increasing the agglutinability of the bacillus. Our experience suggests that this treatment, which almost always gives a satisfactory result, acts by encouraging the multiplication of highly motile forms, which, as we have seen, are more readily agglutinable than elements deficient in motility.

Temporarily non-motile and inagglutinable typhoid bacilli are not infrequently found in the blood or stools of typhoid cases; and although there is no direct evidence on the point, it is conceivable that cases can occur in which the infecting agent is present exclusively in the non-motile phase. If this were so in any particular case, the patient's serum would correspond with our "non-motile" artificial serums, and its agglutination of the motile form (*i.e.* of ordinary good or standard cultures) would be of low grade, or might even be entirely absent.

A case of this kind, if it exhibited the classical symptoms, but yielded no specific bacillus to cultural tests, and if the serum failed to agglutinate reliable cultures of typhoid and paratyphoid bacilli, would be classed as clinical enteric, though bacteriologically and serologically negative. We should like to suggest that it would be well in such rare cases to test the serum with a suspension prepared from a culture of non-motile typhoid bacilli, as well as with cultures of the Gaertner group, which we now know can also cause "clinical enteric."