GRANULAR "O" AGGLUTINATION IN PARATYPHOID B AND TYPHOID FEVERS.

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I. Methods.

The first step of our enquiry into the frequency, specificity and diagnostic value of "granular" (O) agglutination in paratyphoid B fever was to find the best possible reagent for its detection and measurement.

Knowing that formolised diluted broth suspensions of the natural non-motile (O) variant of *Bact. typhosum* "901" are excellent for the diagnosis of typhoid fever, we prepared similar suspensions of the natural "O" form of *Bact. aertrycke* (Glasgow), the chief antigen of which has been shown (Bruce White, 1926) to be identical with the heat-stable somatic antigen of *Bact. paratyphosum* B. We compared these formolised broth suspensions of *Bact. aertrycke* (Glasgow) with living suspensions of the same strain and with suspensions treated with 50 per cent. alcohol to destroy the flagellar antigen. Parallel with these we tested suspensions of the motile, smooth (O.H.) variant of *Bact. paratyphosum* B, both living and after alcohol treatment. Human serums from normal persons and from cases of paratyphoid B fever were employed.

These experiments showed that formolised aertrycke "O" suspensions are first-class specific reagents for the detection and measurement of paratyphoid B "O" agglutination. Their sensitiveness to granular agglutination is on an average slightly less than that of living suspensions, but this is no disadvantage (Gardner, 1929), and since they are dead and preserved, their behaviour in consecutive tests is more consistent than that of any series of freshly made living suspensions. They also have the advantage of complete safety. We have not found it possible to work accurately with living motile suspensions, because the quantitative estimation of two types of agglutination in a single suspension seems to us too difficult and uncertain.

Alcohol-treated suspensions of *Bact. paratyphosum* B grown on agar containing 1 in 800 phenol are of high sensitiveness and excellent quality, and behave in all serological respects exactly as the natural "O" form of *Bact. aertrycke*. Formolised broth suspensions of the latter appealed to us chiefly because we are accustomed to making and handling such suspensions in large quantities.

For the making of "O" suspensions of *Bact. paratyphosum* A, we had recourse to the alcohol treatment of smooth, motile agar cultures, since no

permanent non-motile variant of this organism has yet been isolated. These suspensions we found to react well with the "O" agglutinins of immunised rabbits.

Our technique followed the principles of Dreyer's method (Med. Res. Counc. 1930). A single, selected and well-tested suspension of each type was used throughout the series of experiments (Table I). The readings of granular agglutination in killed suspensions were taken after 24 hours at 52° C.; in living ones, after the same period at 37° C. Floccular agglutination was estimated with formolised broth H suspensions, the results being read after 2 hours at 52° C. The dilution of serum in which the last definite trace of agglutination could be seen was recorded as the observed titre (O.T.) of the serum. In some of the series of observations reduced titres (R.T.) were calculated, by first adjusting the actual end-point reading to the value of "standard" agglutination by means of the appropriate factors in the reduction table, and then dividing the figure by the reduction factor of the suspension (Med. Res. Counc. 1930). This simple calculation will be shown later to offer help in differential diagnosis.

Serums. The serums used for the main experiments were from cases diagnosed as typhoid or paratyphoid B fever. Since blood culture, the best diagnostic method, is seldom practised in this country, the diagnosis rested generally on clinical data and a positive H agglutination test, and we were thereby deprived of the opportunity of examining for granular agglutination any "Widal-negative" serums from bacteriologically proved cases. The majority of the paratyphoid B serums came from the epidemic at Epping in the early spring of 1931.

II. GRANULAR AGGLUTINATION BY NORMAL HUMAN SERUMS (see Table I).

Fifty unselected and unheated specimens of serum from persons not suffering from enteric fever were titrated with the various "O" suspensions at dilutions of 1 in 25 and upwards, to establish the limits of non-specific agglutination.

Table I. *Granular agglutination tests of* 50 *normal human serums.*

	Number of serums showing trace-titres at dilutions of			
Suspensions:	0–25	25–50	50–100	100–200+
Bact. typhosum "O"	31	16	2	1
Bact. aertrycke "O" (para. B "O")	44	5	1	0
Bact. paratyphosum A "O"	50	0	0	0

In Table I it is seen that the typhoid "O" suspension gives higher and more frequent reactions than the paratyphoid B "O," and that paratyphoid A "O" gives no reaction at all at 1 in 25. Evidently, then, the dividing line between normal and specific granular agglutination must be fixed at different levels for the different species of bacteria. This, indeed, had already been done in the case of the typhoid and aertrycke suspensions by previous tests with

normal serums; and the reduction factors for the series of "O" suspensions of the former organism had been fixed at a considerably higher level than those of the latter, in conformity with the practice of the Standards Laboratory, which is to set the levels so that the diagnostic limit may in all cases stand at a reduced titre (observed titre divided by suspension factor) of 10.

Using the factors already thus fixed for the typhoid and paratyphoid B (aertrycke) "O" suspensions (*i.e.* 11 and 6 respectively), we can see what the reduced titres work out at. The observed fine-trace readings are first converted to the values for "standard" agglutination, by halving the dilution figures, since standard agglutination is known to need approximately twice the amount of serum that gives a fine trace. The figures thus obtained are then divided by the sensitivity factor of the suspension. By this method of calculation we find that 49 of the 50 serums have reduced titres of less than 10 for *Bact. typhosum*, and that all the 50 have less than 10 for the paratyphoid bacilli. The one serum which exceeds this limit gives R.T. 15 for typhoid (trace reading at 1 in 320). This is exceptional, and probably indicates a past infection. None of the other serums approaches a R.T. of 10 with any of the suspensions; in fact only one (the highest paratyphoid B figure), exceeds 3.

We see, therefore, that a limit of 10 for the reduced titre of normal serums holds good in this series of tests.

Since paratyphoid A suspensions (alcoholised) are relatively very insensitive to normal agglutination, their range of sensitivity factors would have to be set very low to preserve a diagnostic limit of R.T. 10.

III. Titration of 80 serums from cases of typhoid and paratyphoid B fever (Table II).

Most of the serums were supplied to us in very small quantities, so that we were unable to titrate them against more than a few suspensions. For this reason we concentrated on the "O" reactions, and used "H" suspensions only to check the specific titre, on which the diagnosis in most cases had been based. The observations are recorded in Table II. The figures in the columns under the heading of "O" suspensions represent the last dilution in which a definite trace of clumping was seen after 24 hours at 50–55° C. The numbers, *e.g.* 1000, are abbreviations for dilutions, *e.g.* 1 in 1000. The H titres represent readings of well-marked flocculation without deposit (*i.e.* standard agglutination).

No attempt is made to classify the cases according to the period of the illness, severity, etc., since we had scanty information on these matters. Nor do we know how many of the subjects had been inoculated against enteric fever in the past. *Recent* inoculation may, however, be ruled out.

The main facts that emerge are:

1. *In the* 40 *typhoid serums* the observed (trace) titres for *Bact. typhosum* (O) range from 125 to 5000; the paratyphoid B "O" titres range from 0 to 250;

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Table II.

Typhoid serums							Paratyphoid B serums						
	Titres given with various suspensions				Ratio of granular titres Para. B/Typhoid			Titres given with various suspensions				Ratio of granular titres Para. B/Typhoid	
		Last trace of agglutination						Last trace of agglutination					
No.	Typhosum floccular	Typhosum granular	Para. A granular	Para. B (aertrycke) granular	Observed	Reduced	No.	Para. B floccular (specific)	Typhosum granular	Para. A granular	Para. B (aertrycke) granular	Observed	Reduced
1	1000	2500	125	125	0·05	0·2	41	1000	25	0	40	1·6	5·9
2	500	250	50	125	0·50	1·8	42	900	125	0	125	1·0	3·7
3	1000	5000	125	250	0·05	0·2	43	400	500	35	250	0·5	1·8
4	2500	250	25	50	0·20	0·7	44	600	200	20	80	0·4	1·5
5	250	250	0	25	0·10	0·4	45	600	0	0	60	> 2·0	> 8·8
6	250	250	25	50	0·20	0·7	46	600	25	0	200	8·0	29·2
7	250	500	0	25	0·05	0·2	47	25	0	0	250	>10·0	>36·6
8	2500	2500	50	125	0·05	0·2	48	1500	125	50	500	4·0	14·7
9	500	250	0	0	<0·10	<0·4	49	3000	35	0	40	1·1	4·2
10	250	250	0	0	<0·10	<0·4	50	70	25	0	0	< 1·0	< 3·7
11	125	250	50	125	0·50	1·8	51	1500	90	0	300	3·3	12·2
12	2500	250	0	25	0·10	0·4	52	6000	50	0	100	2·0	7·3
13	250	500	0	50	0·10	0·4	53	5000	125	40	200	1·6	5·9
14	125	500	0	125	0·25	0·9	54	7000	200	25	350	1·8	6·4
15	125	250	0	0	<0·10	<0·4	55	6000	80	0	70	0·9	3·2
16	50000	1000	25	50	0·05	0·2	56	6000	125	40	400	3·2	11·8
17	250	250	0	0	<0·10	<0·4	57	4000	500	40	150	0·3	1·1
18	5000	500	0	50	0·10	0·4	58	4000	50	0	150	3·0	11·0
19	1000	250	0	0	<0·10	<0·4	59	2500	50	0	90	1·8	6·6
20	2500	1000	50	250	0·25	0·9	60	3000	25	0	170	6·8	24·9
21	250	2500	50	125	0·05	0·2	61	1000	50	0	25	0·5	1·8
22	5000	1000	25	50	0·05	0·2	62	3000	0	0	0	—	—
23	1000	2500	50	25	0·01	<0·1	63	500	50	0	50	1·0	3·7
24	1000	2500	50	125	0·05	0·2	64	1500	125	0	80	0·6	2·3
25	2500	2500	50	125	0·05	0·2	65	20000	600	150	400	0·7	2·4
26	500	500	50	50	0·10	0·4	66	3000	50	0	90	1·8	6·6
27	125	1000	0	25	0·02	0·1	67	10000	250	25	300	1·2	4·4
28	250	250	25	50	0·20	0·7	68	900	50	0	50	1·0	3·7
29	0	125	0	0	<0·20	<0·7	69	1500	125	0	125	1·0	3·7
30	250	1000	25	125	0·12	0·5	70	5000	40	0	60	1·5	5·5
31	250	500	50	125	0·25	0·9	71	5000	150	0	400	2·7	9·8
32	0	500	50	250	0·50	1·8	72	1000	350	25	90	0·3	0·9
33	125	500	50	250	0·50	1·8	73	1000	250	0	50	0·2	0·7
34	200	200	30	125	0·60	2·3	74	700	50	0	100	2·0	7·3
35	10	550	0	40	0·07	0·3	75	1000	0	50	250	>10·0	>36·6
36	600	1400	25	125	0·09	0·3	76	1000	0	0	25	> 1·0	> 3·7
37	125	700	0	0	<0·04	<0·1	77	7000	250	40	125	0·5	1·8
38	400	700	25	60	0·09	0·3	78	600	30	0	100	3·3	12·2
39	1500	400	25	125	0·31	1·1	79	7000	250	40	400	1·6	5·9
40	250	175	0	25	0·14	0·5	80	600	600	—	2000	3·3	12·2
	Average (Mode) of Ratios				0·1	0·4						1·6	5·9

on an average a typhoid serum gives with paratyphoid B (O) only one-tenth of its typhoid (O) titre.

2. *In the* 40 *paratyphoid B serums* the typhoid "O" titres range from 0 to 600. The paratyphoid B titres of these serums range from 0 to 2000, and they are, on an average, 1·6 times higher than the typhoid titres.

3. *The paratyphoid A suspension* reacts at serum dilutions ranging from 25 to 125 with half of the typhoid and one-third of the paratyphoid B serums.

4. The agglutination of the typhoid "O" suspensions by typhoid serums is more constant than that of "H" suspensions, but far less specific, as Felix has shown. Paratyphoid B serums, on the other hand, seem to react rather more constantly with the homologous "H" antigen than with the "O"; but we must not forget that the great majority of these serums originated from cases diagnosed exclusively by the ordinary "H" Widal reaction. There is only one paratyphoid B serum in our series, No. 47, in which the "H" titre is below the diagnostic limit, and the "O" titre above it. Unfortunately this case was not proved by blood-culture.

In the light of these observations, we feel that the use of "O" suspensions should help in the identification of paratyphoid B fever after triple inoculation, though we have been unable to get direct evidence on this point.

5. In this connection it is interesting to see what kind of diagnostic information the "O" suspensions alone would have given, if the test for "H" agglutination had not been done; or if the "H" titres, by reason of previous inoculation, could not have been used for diagnosis.

Let us first consider the possibility of "enteric group" diagnosis, without any attempt at differentiation. If, on the basis of Table I, we take as a positive result either a trace of agglutination at 1 in 200 or more in the typhoid suspension, or a trace at 1 in 50 or more in paratyphoid B, the great majority (actually 74 out of 80) of the serums could be diagnosed by the combined use of the two "O" suspensions. The remaining six would have been missed (41, 49, 50, 61, 62 and 76), though all but No. 62 might have been thought suspicious.

Turning now to the question of differential diagnosis by means of "O" suspensions, and examining the ratio of the paratyphoid B to the typhoid observed titres, we find that of the 74 serums which reacted sufficiently with one or other of the suspensions, 50 gave ratios (of unreduced titres) smaller than unity. It would be natural to suppose that this kind of ratio means typhoid fever, whilst its reverse means paratyphoid B. But since nine of the ratios less than unity were obtained with serums known to have come from cases of paratyphoid B fever, this method is clearly unsatisfactory.

The reason is not far to seek why *all* the anomalous ratios are found in the paratyphoid B series, for we have seen that paratyphoid B suspensions react at a definitely lower level than the typhoid suspensions, owing to the initial normal base-line difference (see Table I). The effect of this is to magnify the apparent typhoid titres relatively in all serums, and to create an error in favour of a typhoid diagnosis.

Now the highest ratio in the typhoid series is 0·6, and the lowest in the paratyphoid B series is 0·2. Any serum, therefore, which has a paratyphoid B titre less than 20 per cent. of its typhoid titre can be diagnosed as typhoid; and any serum in which the percentage is greater than 60 can be diagnosed as paratyphoid B. By this test 55 of the 74 serums with diagnostic titres can be placed in their right class. The remainder can only be diagnosed as "group" enteric.

It must not be forgotten that these ratios, and the deductions made from them, only hold good for the actual suspensions used. With any other set of suspensions the figures would be different.

We have, however, a means of making a correction both for the different normal levels and for the variable sensitiveness of suspensions, by making use of the reduction factors for the two types of suspensions.

We have therefore calculated the reduced titre with these factors, and worked out a fresh set of ratios, which are given in the last column of each half of Table II. In this series we see that any ratio less than 0·7 could confidently be diagnosed as typhoid, and any ratio more than 2·3 as paratyphoid. Between these values there is a zone of uncertainty.

In this way we obtain a correct differential diagnosis in the same proportion of cases as before (55 out of 74); but the limiting ratios 0·7 and 2·3 will hold good for any suspensions standardised in series with those employed. To put the matter in a more approximate and practical form: When suspensions of measured sensitiveness are used, if the reduced "O" titre for typhoid is double that for paratyphoid B, the case can be diagnosed as typhoid. On the other hand, if the reduced titre for paratyphoid B is more than double that for typhoid, the diagnosis of paratyphoid B fever is almost certain.

IV. The Specificity of the Somatic ("O") Antigens.

The researches of Felix (1924) and Bruce White (1926) show that the heat-stable somatic antigens of *Bact. typhosum* and *Bact. paratyphosum* B have both specific and group factors. Felix emphasises the latter, and states that in persons inoculated with polyvalent vaccine, a differential diagnosis (by means of the "O" agglutination) will be possible only in exceptional cases, and that the diagnosis of "enteric group" is all that can usually be made. This view seems to us to need modification. Assuming that inoculation, unless very recent, does not appreciably affect the "O" agglutination, our results may be taken as very similar to what would be found in an inoculated group of enteric patients; and a differential diagnosis, on the lines we have indicated, would be obtainable in the majority of cases. This point, however, needs experimental verification.

Bruce White (1926), on the basis of extensive experiments with rabbit immune serums, distinguishes the heat-stable antigens of *Bact. typhosum* from those of *Bact. paratyphosum* B, allowing only "minor and variable somatic cross-agglutination." This emphasis of the specific side stands in contrast with

Felix's view, and is perhaps explicable by the general tendency of the rabbit to produce highly specific immune serums.

Our experiments point to a stronger group factor than Bruce White indicates; but they suggest, on the other hand, the possibility of making more diagnostic use of the specific factor than is done in Felix's method. For the diagnosis of paratyphoid B fever in uninoculated persons the "H" (floccular) agglutination-test, performed with suspensions both of the specific and the non-specific phases of *Bact. paratyphosum* B, is generally sufficient. Our experiments, however, indicate that some cases may develop "O" agglutinins earlier or more powerfully than the "H," as is not uncommonly the case in typhoid fever. If this is so, the "O" test will be a useful supplement. In persons inoculated with triple vaccine, single estimations of "H" agglutination only give a diagnosis if the titre is very high and the inoculation not too recent. The tracing of large excursions of titre by repeated tests gives clear information in many, but not in all cases.

SUMMARY.

The granular "O" agglutination of human serums from eighty cases of typhoid and paratyphoid B fevers has been titrated with carefully selected killed typhoid "O" and paratyphoid B "O" suspensions of high sensitiveness.

In spite of a wide overlapping, due to common factors, a considerable specificity is shown, and this can be utilised in differential diagnosis, especially if due attention is paid to the different levels of "normal" agglutination for the two species.

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POSTSCRIPT

When our MS. was going to press a valuable paper on this subject by J. Smith appeared in this *Journal* (19. IV. 1932, vol. **32**, p. 143) entitled, "The serological diagnosis of typhoid and paratyphoid fevers." The findings are in the main similar to ours, but it is to be noted that he made use of a considerably shorter incubation period (4 hours), and that he records a coarser degree of agglutination, both of which factors render his recorded titres lower than ours. The writer concludes that the use of paratyphoid B "O" suspensions does not appreciably help in the early diagnosis of paratyphoid.