THE SMALL-FLAKING OR "O" AGGLUTINATION OF PERMANENT STANDARDISED "O" SUSPENSIONS OF *B. TYPHOSUS* BY THE SERUMS OF NORMAL, INOCULATED AND INFECTED PERSONS.

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(With 1 Chart.)

CONTENTS.

INTRODUCTION.

During the last few years the researches of Felix (1924, 1 and 2), Burnet (1924) and Felix and Olitzki (1928) have drawn attention to the importance of the small-flaking or "O" agglutination of *B. typhosus* in the diagnosis of typhoid fever. Gardner and Walker (1921) had already made the tentative suggestion that suspensions of non-motile inagglutinable (or dysagglutinable) typhoid strains might give a positive reaction with typhoid-fever serum when the ordinary Widal reaction was negative. The bacilli in suspensions of this kind are what is now known as the "O" type. They are agglutinated by typhoid agglutinating serums very slowly and in minute clumps or flakes, unlike the large fluffy flocculi produced by the bacilli in the motile (H) phase. We now know that suspensions of the same properties can be made artificially by destroying the "H" substance, which is bound up with the flagella, by heating to 100° C. or by treatment with alcohol.

The pioneers of this line of investigation were Malvoz (1897), Smith and Reagh (1903–4) and Walker (1903, 1918), and contributions of much importance have been made by Arkwright (1921), Weil and Felix (1920), Andrewes (1922), Bien (1924) and White (1926).

Although the general position taken up by Felix and Olitzki (1928) in regard to the diagnostic significance of small-flaking agglutination in typhoid

fever may be taken as established, certain of their experimental findings and, more especially, the hypotheses based on them, are open to question. In particular, Felix's view that there is no increase of small-flaking (O) agglutinins in the serum of inoculated persons seemed to demand further investigation. Part of this paper deals with that question, and the remainder is concerned with the technical aspects of the preparation and standardisation of typhoid "O" suspensions, and discussions of the diagnostic limit and sphere of applicability of this type of reaction.

I. THE TYPHOID "O" AGGLUTININS IN THE SERUMS OF NORMAL AND INOCULATED PERSONS.

"O" suspensions of B. typhosus.

Agar cultures of smooth-phase *B. typhosus*, strain "Rawlins," which, as Dr Felix informed me, is rich in heat-stable (O) "antigen" were treated with alcohol (for details see Section II) to destroy the flagellar (H) agglutinable substance. The suspensions were stored without further treatment, and suitably diluted with salt solution for each experiment. Various tests with animal and human sera (see p. 385) showed them to be rich in the small-clumping (O) substance, and devoid of large-flocculating "H" substance. Serologically they had nothing in common with similarly prepared suspensions of a rough *B. typhosus* (strain "Lab.").

Throughout the series of tests described in this section a single "O" suspension was used. Repeated titrations with a stable stock serum showed that it retained its agglutinability unimpaired.

The Serums examined (Table I) were:

(1) A random group of 57 serums sent for the Wassermann reaction. In 47 of these there was no evidence of antityphoid inoculation; in the remaining 10, although there was no direct evidence of inoculation, the results of the ordinary T.A.B. Widal reaction strongly suggested that it had been done.

(2) Six serums from persons known to have been inoculated with T. or T.A.B. vaccine in the last 13 years.

(3) A batch of 11 mental patients who had just been inoculated with T.A.B. vaccine owing to the occurrence of a few cases of typhoid fever in the hospital.

(4) Some cases of typhoid fever, the serums from which were sent to me by various obliging pathologists.

The numbers in the various classes, with some further details, and the results of the tests are shown in Table II.

Agglutination technique.

Dreyer's method of setting up the test was used throughout (macroscopic; drop-measurement; 10 drops of graded serum dilution plus a constant volume of suspension equal to 15 drops). The titres of small-clumping (O) agglutina-

Table I. *Showing the number of typhoid "O" standard units of agglutinating power in the serums of 87 persons.*

1 Stone	0*	24 Ware	0	47 Leslie	3·5	67 Money	2·0
2 Mole	0	25 F. G.	0·5			68 Whit	3·0
3 25i4	0	26 Castle	0·5	48 Read	0	69 Morris	3·0
4 Mrs P.	0	27 Walt	0·5	49 2454	0	70 Casey	4·0
5 B14979	0	28 Read	0·5	50 Savage	0	71 Free	5·0
6 Collier	0	29 5043	0·5	51 B15898	0·5	72 Craw	9·0
7 2693	0	30 Hiles	0·5	52 2454	0·5	73 Reyn	9·0
8 Wyatt	0	31 2552	0·8	53 Shaw	1·0	74 Milt.	18·0
9 Beal	0	32 Rowl.	0·8	54 Lock	1·0		
10 Foulkes	0	33 Monk.	0·8	55 2774	2·0	75 Nurse P.	1·0
11 2953	0	34 2776	0·8	56 Hoop	2·5	76 Cow. B.	6·0
12 Tound	0	35 A. H.	1·0	57 3056	3·0	77 Cow. C.	10·0
13 Anwood	0	36 1702	1·0			78 Cow. A.	15·0
14 H. A.	0	37 B15524	1·0	58 Crone	0	79 E. W.	38·0
15 Form.	0	38 Hunt	1·0	59 A. D. G.	0	80 M. W.	170·0
16 Cairn	0	39 Mr B.	1·0	60 JERW	0·5	81 Brown.	180·0
17 Lewis	0	40 E. F. S.	1·5	61 E. W.	1·0		
18 Whipp	0	41 Shil.	2·0	62 MCR	8·0	82 JLBI	3·8
19 Pull.	0	42 2672	2·0	63 Sykes	9·0	83 J. W.	8·8
20 14711	0	43 3044	2·0			84 Cole	20·0
21 Warren	0	44 2438	2·0	64 Belg.	0·5	85 Mrs G.	80·0
22 Brice	0	45 3065	2·0	65 Bath	1·0	86 E. D.	90·0
23 Law	0	46 Gom	2·0	66 Robins	1·0	87 M. H.	125·0

* Each zero really means "less than 0·5" units, which is the limit of observation. For statistical purposes the zeros are taken as 0·2 units, which probably represents approximately their average value.

Table II. *The strength of small-clumping (O) agglutination of* B. typhosus *in 87 human serums of various groups.*

"O" titre (dilution of trace readings)	Normal. No evidence of inoculation	Inoculation in the past		Recent inoculation (4 weeks ago)	Typhoid fever		Standard "O" units
		Probable	Certain		On clinical & serological evidence	Bacteriologically proved	
0–15	24	3	2	—	—	—	0
20–50	22	5	2	4	1	—	0·5–2·0
55–200	1	2	1	4	1	1	2·5–8·0
250–800	—	—	1	3	2	2	8·5–32
950–3,200	—	—	—	—	1	2	33–120
3,500–10,000	—	—	—	—	2	1	Over 120
Total	47	10	6	11	7	6	—

tion given in Table II were calculated from the readings obtained after 20–24 hours' incubation in the water-bath at 52–53° C. with the tubes half-immersed; the last definite trace of clumping being taken as the end-point.

Explanation of Table II.

The results of the "O" agglutination test with the 87 human sera, are charted in six groups according to the serological history of the case, and in five divisions according to the height of titre. Evidence concerning inoculation was difficult to obtain, and the column of "Normals" may contain some anciently inoculated persons. In the column "Probable inoculation" there are included the persons, mentioned above, whose serum gave a positive Widal (H) reaction either to *B. typhosus* or to *B. paratyphosus* B., or both. They were mostly males who were of military age in 1914–18.

The class of recently inoculated persons had received two doses of Parke-Davis T.A.B. vaccine; the second dose preceded the tests by 4 weeks.

Standard "O" units were calculated from the dilutions in which "standard agglutination" was observed to occur, by dividing the dilution by the agglutinability factor of the suspension, *i.e.* 20 (see later). If one tube of the series showed complete agglutination, and the next only a trace, "standard agglutination" was taken as occurring at an intermediate dilution. A permanent gelatin tube showing "standard agglutination" of dysentery bacilli was used for comparison.

Since the "O" titres given as dilutions in Table II represent the finest readable traces of clumping, the values for "standard agglutination" are proportionately lower; the actual figures being usually in the ratio of about 2 to 1. Thus, when a fine trace is given at 1/100 "standard agglutination" is found at about 1/50. Dividing 50 by 20 we get 2·5 as the number of standard "O" units in this serum.

Discussion of Tables I and II.

In Table II the regular increase of the concentration of "O" agglutinin as we pass from normal persons, through inoculated persons, to cases of typhoid fever, is seen at a glance. Comparing the "Normal" class with recently inoculated persons we find a negative reaction (0 units) in more than half of the normals, but in none of the recently inoculated.

On the other hand, titres of over 2·5 units are found only in 1 out of 47 normals, but in 7 out of 11 of the recently inoculated.

Looking at the figures given in Table I, we see that the total number of units in the 57 cases *not known to have been inoculated* is 37; the average units per serum 0·65, and the range from 0 to 3 units. Similar figures for the 17 cases *known to have been inoculated* either recently or in the past, are: total units 74; average per serum 4·3; range 0 to 18. The average of the latter group is thus nearly seven times that of the former.

Finally, by a more exacting method of statistical analysis we find in the 47 "Normals" an average of 0·7 units and a standard deviation of 0·73. For the recently inoculated class (11 cases) the average is 5·0 units and the standard deviation 4·96. Calculating the probable error of the combined groups we get $\pm$ 1·01. The difference of the two averages, 4·3, is more than four times the probable error, and establishes a probability of 10,000 to 1 that the average of any group of recently inoculated persons will be higher than that of any group of normals.

An objection might perhaps be raised concerning the *recently inoculated class*, in that, apart from the question of inoculation, it is not a truly random group of persons, but a small batch of lunatics in a hospital where typhoid fever was occurring intermittently. It is therefore conceivable that their higher average "O" agglutinins might be due to subinfection, and not to the inoculation. This objection, however, is considerably weakened by the fact

that in 7 tests of uninoculated persons from the same population, all showed normal titres (less than 1 standard "O" unit).

In order to place the matter on firmer ground, a healthy adult was inoculated with T.A.B. vaccine, and his "O" agglutinin carefully titrated at intervals both before and after inoculation. This person had been inoculated with T. vaccine in 1914 and with T.A.B. in 1915. His "H" titre had sunk to about 1 in 200, and his "O" titre stood at 1 in 3. The results are given in Chart 1, and they show that considerable quantities of "O" agglutinin are produced as a result of inoculation with the customary T.A.B. vaccine.

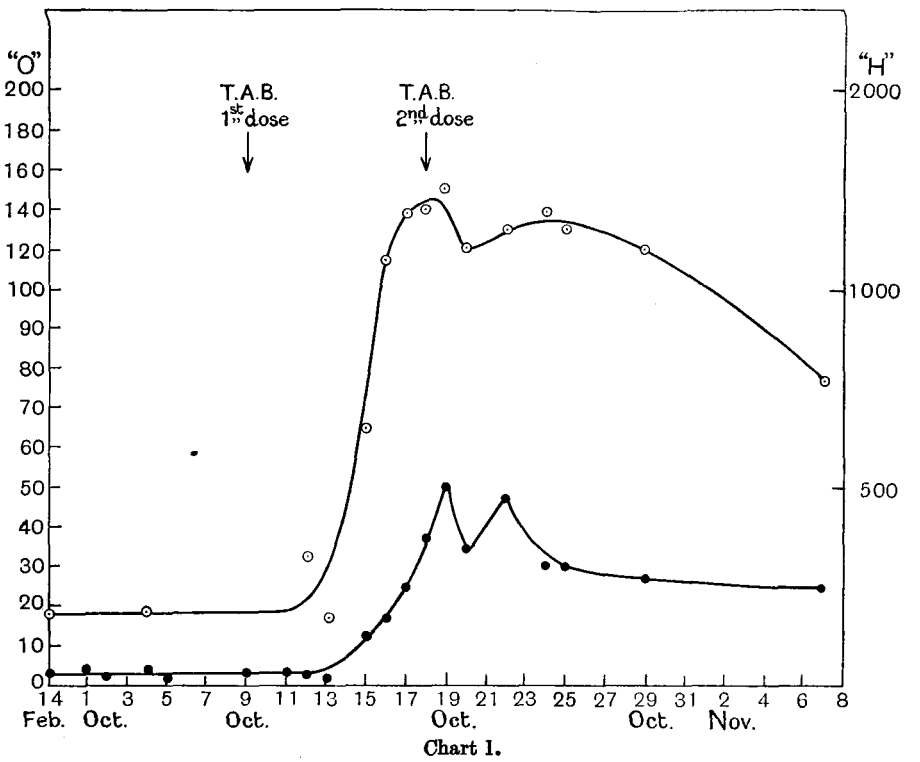

Chart 1.

Curves of "O" and "H" agglutination of *B. typhosus* following triple (T.A.B.) inoculation.
Large-flocculating "H" agglutination = ⊙———⊙ scale on right.
Small-clumping "O" agglutination = •———• scale on left.

In fact, although the "O" curve runs at a much lower level than the "H," its seventeen-fold rise (from 3 to 50) is actually double the eight-fold "H" excursion (from 185 to 1500). In this sense the "O" response might be considered as the more active of the two.

The serums of the 11 recently inoculated persons in Table II were retested 5 weeks after the first test (*i.e.* 9 weeks after the second dose of vaccine). Seven showed a fall of titre, but in 3 of these it was very slight, and the 4 others gave, within the range of experimental error, unchanged readings. The

total number of units in the group fell from 55 in the first test to 38 in the second. Meanwhile the drop of ordinary (H) agglutinin was much sharper; since the mean of 370 (H) units per person in the first test fell to 60 in the second, and all individuals participated in the fall.

The range of titres of "O" agglutination.

The absolute magnitude of the titres found in presumably normal persons is higher than those given by Felix (1924) and by Whitehead (1927). The explanation lies in (1) the great sensitiveness of the suspension used. An exact similar suspension made shortly afterwards was less than half as sensitive (see later). (2) Incubation for 24 hours at 50–55° C., a higher temperature than Felix's and a longer period than Whitehead's. (3) The fineness of the readings taken, *i.e.* the last trace of clumping visible with a watchmaker's lens.

Since the control tubes of the "O" suspension alone were always devoid of granularity, readings as fine as these could be taken without risk of error.

Inoculation in the past.

The average of the titres of the 16 serums in this section is distinctly higher than the normal average. The aggregate standard units of the 16 cases are 29, with an average of 1·8 units per case. These figures are to be compared with an aggregate of units for the 47 normal cases of 28, and an average of 0·5 units per case. Since inoculation gives rise to the production of "O" agglutinins these figures are the kind of thing one would expect from long past inoculation.

Typhoid fever.

All the cases gave a positive Widal (H) reaction, but in one case it was very low (1/25 or less than 5 standard units). This is the serum that showed only 1 unit of "O" agglutination and since it was not bacteriologically proved, it may not have been typhoid fever at all.

In 7 cases the "H" titres were from 50 per cent. to 800 per cent. higher than the "O," and in 6 cases the "O" titres were from 400 per cent .to 4000 per cent. higher than the "H." In 1 case the "H" agglutination was in the diagnostically doubtful region (1/50 or about 9 H units), whereas the "O" titre of 1/1000, or 38 (O) units, was diagnostically conclusive.

It was unfortunately impossible to obtain any serums from bacteriologically proved typhoid cases with negative Widal (H) reactions. Such serums seem to be a great deal less common in this country than in Palestine (Felix, 1924) and in Pretoria (Pijper, 1923).

Diagnostic level of "O" agglutination.

Leaving out of consideration the group "O" agglutination due to Salmonella infections, and considering only typhoid fever proper, we see from Table I that only 1 out of 63 persons not suffering from typhoid fever and not recently inoculated showed more than 8 standard "O" units (trace at 1 in 200). Actually this one case had 9 units.

The borderline, then, between normal and abnormal lies in the region of 10 units. Though an absolute line is impossible to fix, it is legitimate to say that an "O" titre of 10 units very probably means active infection. Even 5 units should arouse suspicion: 20 units are almost diagnostic (the highest recent inoculation gave 18), and 30 may be considered as conclusive.

If inoculation and past enteric infection can be excluded, values below 10 units become so much the more significant.

We must not forget that the "O" agglutinin alone cannot give us the diagnosis of typhoid fever, but only of "enteric group." For the typhoid "O" agglutinin is identical with that of Gaertner's bacillus, and overlaps serologically with others of the Salmonella group.

The theoretical and practical significance of "O" agglutination due to inoculation.

Felix's experience that the serums of inoculated persons do not effect the small-flaking agglutination of living typhoid bacilli at a dilution of 1 in 100, coupled with his view that the "O" substance and antibody are the only really important immunological substances concerned in infection and immunity, led him to the conclusion that inoculation against typhoid fever is worthless. Whitehead (1927) was also unable to detect "O" agglutinins in the serums of recently inoculated persons. This was surprising, seeing that in rabbits the injection of formolised suspensions of smooth typhoid bacilli causes a rich production of "O" agglutinin, and that Goyle and Bryce (quoted by Arkwright, 1927) found "O" agglutinin in the serum of rabbits injected with heat-killed vaccine. But since the experiments described above prove that antityphoid inoculation does stimulate a production of "O" agglutinin in human beings, just as in rabbits, the theoretical ground for Felix's somewhat revolutionary theory disappears. It seems that his observations are sound, but his deductions unjustified. We may accept the fact that, with his technique, the "O" agglutination of inoculated persons practically always fails at 1 in 100, whereas that of typhoid fever patients, almost without exception, exceeds that figure at some stage of the disease. But this does not mean that inoculation has had no effect, nor is there any law by which an antibody must be present in a concentration of more than 1 in 100 before we are entitled to consider it of immunological importance. It is therefore clear that, whatever the significance of the "O" agglutinin may be, we cannot admit the truth of any theory based on its absence from the serum of inoculated persons.

It seems likely that inoculation does not cause, on an average, nearly so great a rise of "O" agglutination as typhoid fever, and therefore the two conditions are, as Felix believes, to a large degree distinguishable. But we have not sufficient data to state the limits to which inoculation may drive up the titre at the height of the curve, and it is therefore necessary to take recent inoculation into consideration in the interpretation of the reaction. We have seen that when the inoculation is long past, the residual "O" titre, though

still on an average higher than normal, can be allowed for in the fixing of the diagnostic limit.

II. The Preparation and Standardisation of "O" Suspensions.

Felix rightly advocates the agglutinability standardisation of "O" suspensions on the ground that strains of *B. typhosus* differ greatly in their strength of "O" substance. We may add that different suspensions of the same strain also differ profoundly in this respect, and not only according to their grossly smooth or rough condition, but even when both seem superficially smooth.

The principles of agglutinability standardisation.

Dreyer's system of agglutinability standardisation (*Med. Res. Council, Spec. Rep. Ser.* 51) was originally based on the tacit belief that the agglutinable substance of a bacillus, and the agglutinin resulting from injection or infection with it are single substances or properties. We now know this to be erroneous. (Walker, 1918; Weil and Felix, 1920; Arkwright, 1921; Gardner and Walker, 1921; Andrewes, 1922; Felix, 1924, 2; Burnet, 1924.) Since all this group of bacteria (and many others) are antigenically complex and variable, it might be thought that standardisation of agglutinability is impossible. But in practice comparatively little trouble has arisen from this cause. In the case of *B. typhosus* the ordinary standardisation measures the quantity of "H" agglutinin in the suspension. The co-existence of the "O" substance in various proportions does not appreciably vitiate the measurements; because the artificial sera used for standardising are much stronger in "H" than in "O" agglutinin, and because the slowness and fineness of the "O" agglutination in formolised broth suspensions (see p. 389) prevent any confusion arising. In the case of the Salmonella, a partly unconscious selection has secured the production of motile (H) and preponderatingly specific suspensions; and it is the specific "H" substance that has been standardised.

Nevertheless, the underlying principle of agglutinability standardisation clearly needs re-statement in the following terms: *An agglutinability standardisation can only deal with a single agglutinable substance.*

Preparation of "O" suspensions of B. typhosus *by the alcohol method.*

The method of Bien (1924) of making permanent suspensions for the Weil-Felix reaction was recommended to me by Dr Felix, and has proved perfectly satisfactory.

The culture must be morphologically and serologically smooth. *B. typhosus* "Rawlins" was used in my experiments. Several other strains were found equally good, including three that were opened after having been sealed up in agar stab-culture for 9 years.

Roughness, partial or complete, makes a strain entirely unsuitable for use.

Cultivation. The bacillus is grown on a moist agar surface: in Roux bottles, if a considerable quantity of suspension is required. The layer of agar should be fairly thick, to prevent drying.

Suspension. After 18–24 hours' incubation at 37° C. the growth on each Roux bottle is washed off with about 20–40 c.c. of normal saline solution containing 0·5 per cent. phenol. The suspension is then poured into a measuring cylinder of suitable capacity, and one half of its volume of absolute alcohol is gradually added, while the mixture is stirred with a glass rod. Thus to each 40 c.c. of suspension, 20 c.c. of alcohol is added, making a 33 per cent. alcohol concentration. The cylinder is then capped or plugged with wool and allowed to stand for 12–24 hours at 37° C., for the deposition of solids. At the end of this time the supernatant fluid is poured off and bottled. It constitutes the stock "O" suspension, which appears to keep indefinitely, and has only to be diluted with saline for use. It is essential that the suspension be thick enough to stand a dilution of 1 in 6 or thereabouts, for the concentration of the alcohol in the test-mixtures might otherwise be too high. A convenient density of the diluted suspension is about twice or three times that of the average "standard agglutinable" culture, *i.e.* equivalent to a suspension of about 600,000,000–900,000,000 per c.c. of *B. typhosus* or *B. coli.* A simple way of ascertaining the necessary dilution is as follows. Take 6 dwarf test-tubes of approximately equal diameter. In 5 of them make progressive dilutions of the alcohol suspension, 1 in 1, 1 in 2, 1 in 4, 1 in 8, and 1 in 16. Into the 6th pour some standard agglutinable culture, or any other suspension of the desired density. Match the tubes by indirect artificial light against a dark background, and so ascertain the dilution necessary.

Quality of suspensions.

The large-clumping (H) substance of *B. typhosus* seems always to be completely destroyed or inactivated by treatment with alcohol. But one suspension of *B. paratyphosus* B thus prepared in this way reacted to a slight degree with the homologous "H" agglutinin in the form of scattered fluffy flocculi. This evidently meant that the "H" substance was incompletely destroyed.

The uniformity of "O" suspensions made in this way is all that could be desired, and there is no tendency to spontaneous flocculation.

Preparation of suspensions by the boiling method.

The "H" substance is destroyed by heating simple saline suspensions of smooth *B. typhosus* to 100° C. for 2 hours. It is unnecessary to describe the details, since the procedure is of the utmost simplicity. The suspensions are stored in the concentrated state with a few drops of chloroform for preservation, and suitably diluted (as above) for use.

The suspensions used in this series of experiments.

(1) Alcohol-treated *B. typhosus* "Rawlins" ... T. Raw. Alc.
Supposed antigenic constitution ... ... "O"

(2) Boiled *B. typhosus* "Rawlins" ... ... T. Raw. Boiled
Supposed antigenic constitution ... ... "O"

(3) A formolised broth suspension (standard culture) of *B. typhosus*, strain "Hopkins," smooth, motile ... ... T. Hop. Form.
Supposed antigenic constitution ... ... H (O)

(4) A formolised broth suspension of *B. typhosus* "Rawlins," smooth, motile ... ... T. Raw. Form.
Supposed antigenic constitution ... ... H (O)

(5) A formolised broth suspension of *B. typhosus* "strain Lab.," motile, rough (or semi-rough) ... ... T. Lab. R. Form.
Supposed antigenic constitution ... ... H (R)

(6) An alcohol-treated suspension of *B. typhosus* "strain Lab.," motile, rough (or semi-rough) ... ... T. Lab. R. Alc.
Supposed antigenic constitution ... ... R

(7) A boiled suspension of the same ... ... T. Lab. R. Boiled
Supposed antigenic constitution ... ... R

This last suspension was not sufficiently dispersed to be used in agglutination tests. After incubation it showed almost complete spontaneous flocculation. The effect of reducing the salt content was not investigated.

The results of the cross-agglutination tests are embodied in Table III.

Table III. *Agglutination of formolised, alcoholised and boiled suspensions of smooth and rough strains of* B. typhosus *by the corresponding serums.*

	Names and theoretical antigenic composition of suspensions					
Serums	T. Hop. Form. H (O)	T. Raw. Alc. O	T. Raw. Boiled O	T. Lab. R. Alc. R	T. Raw. Form. H (O)	T. Lab. R. Form. H (R)
T. Raw. Form.	20,000 L	15,000 S	5,000 S	0	*20,000* L	20,000 L
T. Raw. Alc.	5,000 L 20,000 S	*40,000* S	7,000 S	0	—	—
T. Raw. Boiled	600 L 15,000 S	50,000 S	*10,000* S	0	—	—
T. Lab. R. Form.	3,500 L	50 S	10,000 S	250 S	5,000 L	*5,000* L
T. Lab. R. Alc.	30,000 L	100 S	50,000 S	*2,000* S	—	—
T. Lab. R. Boiled	12,000 L	200 S	5,000 S	100 S	—	—

Titres all represent readings after 24 hours at 52° C. H = large-flocculating (flagellar) substance. O = small-clumping (body) substance. R = rough (body) substance. H (O) and H (R) = flagellar substance with smooth or rough body substance. L = large flocculation. S = small clumping.

Serums were made by injection into rabbits of 2–4 doses of the suspension at weekly intervals. The alcohol and boiled suspensions of the Rough strain

(see below) needed 4 doses, the Smooth strain only 2, to produce adequate titres. It may be mentioned in passing that normal rabbit serums gave traces of agglutination with the alcohol-treated suspension T. Raw. Alc. 1 at 1/5 to 1/10. A boiled suspension was clumped in an extremely imperfect manner up to 1 in 200 by all 6 normal serums tested. The alcoholised rough suspension was not influenced at 1/5.

Discussion of Table III.

The two columns on the right hand exhibit the already well-known serological homogeneity of motile smooth and motile rough strains of *B. typhosus*, in so far as the customary large-flocculating "H" substance is concerned.

The columns headed T. Raw. Alc. and T. Lab. R. Alc. demonstrate the great difference between the two strains after treatment with alcohol, which destroys or inactivates the "H" substance. The alcoholised rough suspension contains nothing capable of reacting with any of the sera made from the smooth strain, however treated before injection; and the alcoholised smooth suspension is very feebly affected by any of the rough-strain serums. In present-day terminology the rough strain contains no "O" substance, and the smooth strain very little R.

The effect of boiling, shown in the third column of the suspensions, is clearly not the same as that of alcohol treatment: for the boiled smooth suspension reacts strongly with the R as well as with the "O" serums. But this suspension was, as we have seen, rather unstable and it should be remembered in this connection that the boiled rough suspension was so increased in flocculability that it had to be rejected. The boiling, then, appears to unmask a destabilising substance (? lipoid) in the bacilli and so to speak "roughen" them, perhaps by removing stabilising protein. On the other hand, alcohol treatment stabilises the suspension; perhaps by dissolving away lipoids, as in Bruce-White's (1927) experiments with *B. dysenteriae* (Sonne).

From the failure of serum T. Raw. boiled to clump the alcoholised rough suspension we have to infer that the boiled smooth (Raw.) culture produced no rough agglutinin, and it might be deduced from this that what seemed to be its induced "roughness" was illusory, if it were not for the fact that the boiled true rough suspension itself only succeeded in producing very feeble R. agglutinin. We can at any rate say that as the result of boiling, but not of alcohol treatment, our smooth bacillus acquired some of the characters of the rough variant. Whereas alcohol turns an HO into a relatively pure "O," boiling seems to turn it into O (R). If this is so, it presumably possessed the R element from the first, in a masked condition.

However this may be, since the boiled Rawlins suspension is seen to be weaker in "O" substance than the alcoholic Rawlins, and since it also proved to be less sensitive to the small-clumping action of typhoid fever serums, the alcohol method was considered preferable for the preparation of standardised "O" suspensions for routine work.

Some technical points.

Temperature of incubation. Comparative tests at 37° C. and at 52° C. were done with the T. Raw. Alc. suspension used above. After 2 hours the 37° C. test showed very feeble traces of clumping up to 125, whereas the test at 52° showed strong traces up to 1000 and a weak trace at 2500. After 24 hours' incubation both series had progressed to 5000 (traces); but whereas at 52° complete flocculation went up to 2500, at 37° it only reached 1000. Moreover, inhibition of agglutination, which was just noticeable at 1 in 25 in the 52° series, could be traced up to 1 in 125 at 37°.

It is clear therefore that 52° C. induces more rapid and complete clumping than 37°, and that the end-point after 24 hours is sharper, and slightly higher at 52° than at 37°. 52° is therefore the temperature of choice.

Period of incubation.

The reaction proceeds so slowly that, even at 52°, the customary observation periods of a few hours are quite inadequate. The tests must be left overnight in the water-bath, in which time (18–24 hours) the reaction is practically complete. Half-immersion of the tubes is desirable, since it promotes circulation of the fluid.

Living suspensions.

A certain number of tests have been done with living agar suspensions of T. Rawlins. Their great disadvantage, apart from their infectiousness, is that coarse flocculation (H) and fine clumping (O) occur simultaneously in the same fluid, and have to be distinguished. Although with suspensions of the proper opacity the distinction is usually not very difficult, and with practice may even become tolerably accurate, there is clearly a much greater chance of error than when pure "O" suspensions are used. In the case of *B. enteritidis* (Gaertner) I have not found it possible to distinguish with certainty between the small and the large clumping in living or in formolised broth suspensions.

Standardisation of suspensions.

Having satisfied ourselves that alcohol suspensions of typhoid Rawlins (and most of our strains) in the smooth phase contain the "O" substance in a relatively pure state, we can apply to them the method of agglutinability standardisation hitherto used only in measuring the "H" agglutinability of formolised suspensions.

Method.

A good well-tested "O" suspension is chosen as prime standard. A new suspension that we wish to standardise is tested against the standard suspension repeatedly in parallel series by means of a suitable agglutinating serum, and a ratio of sensitiveness of the two suspensions is calculated (details of the method are given in *Medical Research Council, Special Report Series*, No. 51).

Thus, if the agglutinability of the prime standard suspension be called 1, and if the new suspension is agglutinated up to a 50 per cent. higher titre than the standard suspension the agglutinability factor of the new suspension is 1·5.

But the actual magnitude of the factor chosen for the prime standard suspension depends on the degree to which it is agglutinated by normal serums. Earlier in the paper (p. 381) it is shown that at 1 in 200 practically no normal serum gives strong ("standard") clumping with the particular suspension used, which happens to be our prime standard. So if we fix its agglutinability factor at 20 and apply Dreyer's method of calculation we get 200/20, *i.e.* 10 as the number of standard "O" units that can be taken as indicating a high probability of infection. The advantage of having 10 units as the diagnostic limit is simply that it conforms with the 10 units limit previously fixed in a similar manner for the various standard agglutinable cultures of the typhoid-coli group.

Table IV. *Agglutinability standardisation of a new typhoid "O" suspension against the arbitrarily chosen standard suspension.*

Serums	Titres at which "standard" agglutination was given by: Standard suspension	New suspension	Ratios of agglutinability
1st standardisation, June, 1928.			
T. Raw. Alc.	25,000	12,000	0·48
„	30,000	13,000	0·43
T. Raw. Boiled	70,000	28,000	0·40
N. Human 2776	25	10	0·40
„ 3065	50	25	0·50
„ 2552	25	10	0·40
„ Gomm	60	20	0·33
„ Rowlson	25	10	0·40
		Mean ratio	0·42
2nd standardisation, September, 1928.			
T. Raw. Alc.	24,000	12,000	0·50
„	22,000	10,000	0·43
T. Raw. Boiled	50,000	25,000	0·50
„	50,000	22,000	0·44
		Mean ratio	0·47

In routine work small differences of the agglutinability of suspensions are of little importance, for they fall within the technical error of the test. But it is another matter when the differences are large. For instance, in the comparison of two suspensions given in Table IV the second suspension was less than half as sensitive (8/20) as the first. A repetition of the test 3 months later gave substantially the same ratio. The slight drop in the titres is more probably attributable to the serum than to the suspensions.

Standardisation not only secures that the suspension used for the test contains an adequate quantity of the necessary agglutinable substance, but it provides the necessary correction for the quantitative fluctuation of that substance in different suspensions.

Selection and preliminary testing of cultures.

A typhoid strain that grows with uniform turbidity in broth and in circular, smooth, moist colonies on agar should be selected. The very best strains, containing a great deal of "O" substance usually flocculate incompletely in the higher dilutions of ordinary (HO) agglutinating serum. This is because the "O" agglutinin in the serum is "diluted out" long before the "H," and there are many practically pure "O" bacilli in the suspension, which are not clumped by "H" agglutinin. Certain strains or colonies may prove to be weak in "O" substance without being obviously rough. Close examination of such strains will reveal a lack of pure smoothness. The colonies are not quite what they ought to be, and the growth in broth during the first few hours is finely granular. They are, in fact, intermediate between rough and smooth.

From a strain which, in the bulk, is rich in "O" substance, it is easy to obtain substrains that are lacking in it. For instance, *B. typhosus* "Rawlins" was grown in broth for 2 weeks and then plated out. Although no truly rough colonies were seen, a number were more opaque and less smooth and regular than usual. In broth they gave a granular growth after about 5 hours, though most of them became more or less uniformly turbid in 24 hours. From two of these, and from one opaque pure smooth colony (5 hours broth turbid), alcoholised agar suspensions and formolised broth cultures were prepared. Their agglutinable substances were then estimated with (1) a stock standard agglutinating serum (H titre 5500), (2) a serum made with an alcoholised suspension (O titre 20,000). The results were:

Smooth colony "H" agglutination 2500	"O" agglutination	20,000
Semi-rough col. 1 "H" agglutination 7500	,,	5,000
Semi-rough col. 2 "H" agglutination 8000	,,	50

The figures speak for themselves. It is well known that old stock cultures tend towards roughness, which may become irreversible. It appears that "lack of 'O' substance" and "roughness" are the same thing.

III. CAN FORMOLISED BROTH SUSPENSIONS BE USED FOR DETECTING AND ESTIMATING THE SMALL-CLUMPING (O) AGGLUTININ?

It is stated by Felix that formalin so strongly inhibits the agglutination of the "O" substance, that formalin-treated suspensions cannot be used for estimating "O" agglutinin. He used strong formalin, and a temperature of 37° for incubating his tests. With our technique, however, better results are obtained. If an "O" agglutinating serum is tested with a formolised broth suspension (*e.g.* a standard agglutinable culture) made with a strain of bacillus containing plenty of "O" substance, and if the tests are incubated at 52° C. for 24 hours, a perfectly definite fine-clumping reaction takes place up to, or nearly up to, the full "O" titre of the serum. If the suspension is relatively thin, the fine clumping may be extremely fine, and will need a hand-lens for reading.

If such a suspension be boiled for 20 minutes, it ceases to be capable of large (H) flocculation, but it retains the ability to be very finely clumped by "O" sera. The clumping, however, is irregular, muddy and difficult to read.

Two examples of the small clumping of formolised broth cultures may be cited.

Serum T. Raw. Alc., made by injecting a rabbit with an alcohol T. Rawlins suspension, agglutinated a standard culture, *i.e.* a *formolised broth suspension*, up to 5000 in large flocculi (H), and then further up to 20,000 in minute clumps (O). Its homologous "O" suspension was finely clumped up to 40,000.

Serum E. W. (typhoid fever), agglutinated the (formolised) standard culture in large flocculi (H) to 50, and then in minute clumps (O) up to 500. The pure "O" suspension, T. Raw. Alc. (1), was finely clumped to 1000. It is to be remembered that T. Raw. Alc. (1) is a specially sensitive suspension.

These examples show that with 24 hours' incubation at 52° C. suitably prepared 0·1 per cent. formolised broth cultures can be used for estimating "O" agglutinin. I have however not found it possible to discriminate between large flocculation and small clumping when they occur in the same tube. This may be due to the thinness of the suspensions, which are prepared for the ordinary Widal test. For in a thin suspension containing, say, 75 per cent. H (O) bacilli and 25 per cent. "O," the large clumping uses up all the H (O) and entangles many of the "O," and there are not enough "O" bacilli left to make visible "O" clumps. The utility of such suspensions is therefore limited to the estimation of "O" agglutinin in serums or in dilutions of serum that contain no "H" agglutinin. But it should be emphasised that, before using a formolised suspension for this purpose, it must be proved to contain sufficient "O" substance, and it should be at least roughly standardised for its "O" agglutinability. The excellence of a suspension for ordinary "H" agglutination is no guide, since first-rate large-flocculating suspensions can be made with partially rough motile cultures which, as we have seen, are deficient or even entirely lacking in "O" substance.

Although weakly formolised broth suspensions are, as we have just seen, capable of reacting with "O" agglutinin, the reaction is distinctly less clear and less easy to read than that of alcohol suspensions, and the use of the latter whenever possible is to be recommended.

The place of the "O" agglutination test in diagnosis.

If inoculation can be positively excluded, the estimation of "O" agglutinin is only called for when the customary H Widal is negative or doubtful, *i.e.* (1) in the cases, apparently uncommon in England, in which no appreciable "H" titre develops at all and (2) in the early or late stages of some cases in which a diagnostic "H" titre has not yet developed or has come and gone. An adequately high "O" titre will then give the diagnosis of enteric group infection. It is, however, not possible to identify the infecting species by this test, because the "O" substance is not specific.

As regards inoculated persons, we do not accept the view put forward by Felix that the method of estimating the rise or fall of "H" agglutinin by repeated tests is essentially fallacious, although we admit that it is not always easy to interpret. The view that non-specific T. curves of diagnostic magnitude occur as the result of non-enteric infections (Anamnestic reaction) does not seem convincingly proved. Evidence against it is strong (Perry, 1918; Topley, Platts and Imrie, 1920; *The Official History of the War, Medical Services; Pathology*). Moreover, the figures given and quoted by Felix (1924, 2) àre open to more than one interpretation. The subject is too intricate for further discussion here. But, in spite of these points of difference, we fully agree that the "O" agglutination test is capable of great service in proving the enteric nature of infection in persons whose residual "H" agglutinins complicate the conventional Widal reaction. In fact it seems likely to prove an easier and more reliable test than the tracing of "H" agglutination curves.

Felix suggests the use of two suspensions for routine typhoid agglutination test, *i.e.* a formolised or phenolised "H" suspension, and an alcoholised "O" suspension. With this we are in substantial agreement, making however the reservation that under present conditions in England the ordinary "H" Widal reaction seems usually adequate. The "O" test need only be applied as a second line of enquiry.

Method for the combined "H" and "O" test.

The best method of performing the combined "H" and "O" agglutination test is a controversial matter. A single dilution of serum, *e.g.* 1 in 100, is rgearded by Felix as fulfilling all requirements and as counterbalancing the extra suspension with which the busy pathologist is burdened. But we see no reason to depart from the generally accepted view that single tube methods are dangerous. Unusually high inhibition of agglutination; the chance dirtiness of a tube; a single error of measurement; any of these may vitiate the test. There is safety in numbers. Difficulty also arises in deciding what (single) dilution is suitable, seeing that suspensions vary in sensitiveness. If 1 in 100 is right for a particular suspension, it is wrong for more sensitive and less sensitive suspensions.

But on the other hand it is quite clear that complete titrations are unnecessary in the double test. A method should give the maximum of information with the minimum of labour. We feel that it is considerably safer and more informative to test each suspension (H and O) in at least two serum dilutions, *e.g.* 1 in 100 and 1 in 400. With the two necessary controls this makes six tubes in all for the combined test. By noting the degrees of flocculation in the two tubes of each series an estimation of standard units of agglutinating power can be made. Three grades of readings are sufficient, *i.e.* total (*t*), standard (*s*) and fine trace (*tr*). Roughly speaking in both "H" and "O" agglutination "total" takes 1·5 times the amount of serum needed to give "standard," and fine trace takes only 0·5 times that quantity. Therefore, if

the suspensions used for the "O" test, for example, have a factor of 10, standard "O" agglutination at 1 in 100 gives 10 standard "O" units. Total agglutination at the same dilution gives 15 units, and trace gives 5. At 1 in 400 the values are quadrupled, *i.e.* "total" 60 units, "standard" 40, and "trace" 20. Thus, with this suspension of factor 10, the least trace at 1 in 100 is suspicious; "Standard agglutination" is on the borderline and "total" is almost conclusive. If "O" suspensions of agglutinability factor greater than 30 or less than 5 are used, the two dilutions must be increased or decreased respectively in order to include the critical range of readings. The two dilutions suggested (100 and 400) will also give an adequate range of readings with the customary "H" suspensions (*e.g.* standard agglutinable cultures). When not pressed for time the pathologist will doubtless extract further information by titrating both agglutinins to the end-point. For instance, Felix's prognostic use of the height of the "O" titre merits wider investigation.

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Summary.

(1) The concentration of small-clumping (O) typhoid agglutinin in the serums of a group of recently inoculated persons was found to be, on an average, distinctly higher than that of a group of uninoculated persons.

Additional proof that this is the result of the inoculation is given by tracing the curve of production of "O" agglutinin in a healthy adult immediately after inoculation.

(2) The serums of healthy persons show a moderate but extremely variable power of clumping typhoid "O" suspensions. Expressed as a titre, the normal limit varies between 1 in 50 and 1 in 200, according to the sensitiveness of the suspension used. Expressed in standard units of "O" agglutinating power the normal limit is 10 standard "O" units.

(3) Methods of preparation of permanent suspensions for the estimation of "O" agglutinin are discussed and Felix's recommendation of the alcohol method is confirmed. The agglutinability standardisation of suspensions of this kind is described.

(4) Broth suspensions treated with 0·1 per cent. formalin, such as are used for the ordinary Widal reaction, are shown to be capable of detecting "O" agglutinin, but to be less suitable for its estimation than alcohol suspensions.

(5) The place of small-clumping "O" agglutination in the diagnosis of typhoid fever is discussed, and a method of performing the combined "H" and

"O" test in inoculated persons is suggested, wherein a compromise is effected between the customary quantitative measurement and Felix's qualitative "one-dilution" method.

ADDENDUM.

My attention has been called by Dr Felix to an important matter which had escaped my attention. The majority of the sera of uninoculated persons, Nos. 1 to 57 in Table I, had been heated to 56° C. for 1½ hours before being tested. The unheated sera were Nos. 14, 15, 40, 47 and all from 58 to the end. Unheated also were the seven sera mentioned on page 380, line 1. Since "O" agglutinin is weakened by heat, allowance must be made for this in assessing the figures. The few experiments I have been able to do indicate that the loss of titre at this temperature and time is of the order of 20 per cent.; a reduction which does not vitiate the conclusions I have drawn.

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