THE USE OF PRESERVED BACTERIAL SUSPENSIONS FOR THE AGGLUTINATION TEST.

WITH ESPECIAL REFERENCE TO THE ENTERIC FEVERS AND TYPHUS FEVERS.

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THE following is a report on investigations which were commenced four years ago. The results were always uniform; nevertheless, in order that they might be checked on as much material as possible and as they were contrary to views generally accepted for many years, we postponed publication.

The primary purpose of the investigation was to decide whether it was possible or not to use suspensions of killed bacteria, preserved in the usual way, for the tests in qualitative receptor analysis. It had been proposed to use this qualitative method by one of us (Felix, 1924, 1 and 2), but it was explicitly emphasised in these papers that it was not possible to apply this method without using suspensions of living bacteria. This seemed to be one of the reasons why the method was used by various research workers with success, but was not considered suitable for routine clinical diagnosis.

It may be stated here, that Burnet (1924), working on typhoid patients in Australia and applying the newer knowledge with regard to the double type of antigens and antibodies, observed independently the greater part of the facts established by Felix. The clearness with which Burnet carried out his observations is especially worthy of praise, since the technique which he applied was not very reliable and since the smallness of his clinical material did not permit him to reach such definite conclusions as Felix was able to reach in his work in Palestine.

During the past four years we have had the opportunity of applying the qualitative method on a great deal of clinical material. This experience enables us to state with certainty that the qualitative receptor analysis indeed affords those advantages which Felix has claimed for it.

Now, as is well known, there are several reasons for preferring the use, for routine purposes, of bacillary suspensions, killed and preserved in bulk, in place of newly made suspensions of living bacteria. As a matter of fact for many years there has been an almost general consensus of opinion that such preserved suspensions are not only of equal value but are even superior to fresh suspensions of living bacteria for all the purposes of agglutination tests. Since broth cultures preserved with formaldehyde were recommended by

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Proescher (1902) and the "diagnosticum" preserved with phenol was advised by Ficker (1903) these methods have been universally adopted. The most refined expression of this technique is the method developed by Dreyer (1906 and 1909) and his co-workers.

It would lead too far and is, moreover, superfluous here to quote from the many textbooks and manuals in all countries, to show that no doubt appears to exist that bacteria killed in one way or another can be taken to replace completely the use of living bacteria in the agglutination test. One quotation from the Reports of the Committee upon Pathological Methods, *Medical Research Council Special Report Series* No. 51, 1920, may serve as an example. It reads (p. 113): "Dead bacteria are agglutinated as well as living ones, and there are two advantages in their employment. They entail less risk to the worker, and emulsions can be prepared in bulk and stored, after addition of a preservative, thus effecting not only a saving of time, but greater constancy of results."

But there are two further reasons for this preference in the case of the qualitative receptor analysis.

1. The reliability of this method depends to a greater extent than that of the quantitative methods hitherto used upon the composition of the medium as influencing the agglutinability of the bacteria of the typhoid-paratyphoid group. The change in agglutinability in these bacterial species as well as in the *B. proteus X* strains must be regarded as a phenomenon of variability dependent upon the relative development of their two antigenic components, the labile H and the stable O antigen. The same factors—namely, the reaction (*p*H) of the agar medium, its sugar content and humidity—which have been recognised by various workers as the sources of error in the typhus agglutination with *X* 19, likewise influence the agglutinability of *B. typhosus* and the Salmonellas. In the case of typhus fever the undiminished sensitiveness of the stable O antigen of the culture used, is the only decisive factor, as the typhus patient's serum contains invariably the stabilotropic O agglutinin only. In enteric fevers the unimpaired reactivity of both the O and H antigens of the cultures used is of equal importance, as these patients' sera may contain both the stabilotropic O and the labilotropic H agglutinins. No further details on this subject can be given here. It will be sufficient to state that the absolute need of close observation of certain conditions laid down for the growth of the cultures to be used (Felix, 1924, 1 and 2), may, in a clinical laboratory, cause certain difficulties in routine work.

2. The rough variation, as is known from the work of Arkwright (1921), also causes greater difficulties in this method, since the abundant humidity of the agar essential to the development of the H antigen seems to favour the formation of rough variants.

Great efforts were, therefore, made to decide whether it was possible to employ suspensions preserved in the usual way for this method. Bacteria killed by heating were *a priori* excluded from consideration, since sufficient evidence has already been brought forward to show that the agglutinability

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of bacteria possessing the double type of antigen is affected by heating even by temperatures as low as 50°–55° C.

The Ficker diagnosticum was the first to be tested. It is the most widely recognised preserved emulsion on the Continent; it has been manufactured by Merck for more than 20 years and is said to contain 0·5 per cent. phenol as preservative. The result was as definite as it was astonishing: in the case of the labilotropic H agglutination the sera of typhoid patients, rabbits and horse immune serum all reacted, without exception, as rapidly and as strongly with the diagnosticum as with the fresh suspension of living bacteria. But, again without exception, the stabilotropic O agglutination failed completely.

Table I. *Ficker diagnosticum* (Merck) *and fresh suspension of living* B. typhosus.

Serum	Type of aggluti-nation	Ficker diagnosticum (Merck) *B. typhosus*. Agglutination titre. Reading after 2 hours	24 hours	Fresh suspension of living *B. typhosus*, strain 901. Agglutination titre. Reading after 2 hours	24 hours
Typhoid patient 91, 10th day of illness	*s*	0	0	200	1,000
Rabbit 19 (*B. typhosus* 100° C.)	*s*	0	0	10,000	20,000
Rabbit 31 (*B. enterit.* Gärtner 100° C.)	*s*	0	0	10,000	20,000
Typhoid patient 127, 10th day of illness	*l*	1,000	1,000	1,000	1,000
Rabbit 26 (*B. typhosus* 60° C.)	*l*	20,000	20,000	20,000	20,000
Horse (anti-typhoid serum, Sächsisches serum work, Dresden)	*l*	100,000	100,000	100,000	100,000

Total volume 1 c.c. Incubation 2 hours at 37° C., then at room temperature. *s* = small flakes (= stabilotropic). *l* = large flakes (= labilotropic). Titre 0 = a negative result in dilution 1 : 50.

Table I shows such an experiment. It does not need any explanation. Whereas the slightest traces of H agglutinin were always detected by the diagnosticum, O agglutinins even with a titre of 1 : 20,000 against the living bacteria could not be demonstrated by the diagnosticum even in a concentration of 1 : 50.

Both in the case of *B. typhosus* and *B. paratyphosus A and B*, the results with Ficker's diagnostica (Merck) were invariably the same.

As the diagnostica used were already one year old, age was thought to be the cause of the failures—although it seemed paradoxical that only the so-called stable O component of the bacillary antigen should have been destroyed, while the labile H component was preserved in its full capacity of reaction. But it was found that the behaviour of suspensions newly prepared from our laboratory strains and preserved in the usual way with 0·5 per cent. phenol and 1 per cent. formalin was very similar.

Tables II and III show the difference in agglutinability of suspensions of *B. typhosus* preserved with phenol and formalin respectively as compared with suspensions prepared from the same batch of agar and preserved simply in saline in the cold room. A fresh saline suspension of the same strain prepared immediately before the agglutination test was put up, served as a further control. The density of the suspensions was so chosen that in the agglutination

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test each tube contained 0·5 c.c. serum dilution and 0·5 c.c. suspension. The concentration of phenol and formalin in the actual agglutination tubes was, therefore, 0·25 per cent. and 0·5 per cent. respectively.

As far as the labilotropic H agglutination is concerned no trace of an unfavourable influence of phenol or formalin could be detected. Velocity and

Table II. *Suspensions preserved three days in saline without and with phenol* (0·5 per cent.) *compared with a fresh suspension* (B. typhosus 901).

			B. typhosus 901: Suspension preserved 3 days in saline			Suspension preserved 3 days in saline +0·5 % phenol			Fresh suspension in saline		
Serum *B. typhosus*	Serum dilutions	Type of aggluti-nation	2 hours	6 hours	24 hours	2 hours	6 hours	24 hours	2 hours	6 hours	24 hours
Rabbit 19	1 : 200	*s*	+ + +	+ + +	+ + +	–	(±)	±	+ + +	+ + +	+ + +
(pure O	1 : 2,000		+ ±	+ +	+ + ±	–	–	±	+ ±	+ + ±	+ + +
serum)	1 : 5,000		(±)	+	+ ±	–	–	(±)	±	+	+ ±
	1 : 10,000		–	(±)	(±)	–	–	–	–	(±)	±
	1 : 20,000		–	–	–	–	–	–	–	–	–
Horse (in	1 : 20,000	*l*	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
the dilu-	1 : 50,000		+ ±	+ ±	+ ±	+ ±	+ ±	+ ±	+ ±	+ ±	+ ±
tions used	1 : 100,000		±	±	±	±	±	±	±	±	±
a pure H	1 : 200,000		–	–	–	–	–	–	–	–	–
serum)											
	Saline control		–	–	–	–	–	–	–	–	–

+ + +, + +, +, ± = varying degrees of agglutination, estimated with the naked eye. (±) = traces, estimated by means of a magnifying lens.

Table III. *Suspensions preserved ten days in saline without and with formalin* (1·0 per cent.) *compared with a fresh suspension* (B. typhosus 901).

			B. typhosus 901: Suspension preserved 10 days in saline			Suspension preserved 10 days in saline +1·0 % formalin			Fresh suspension in saline		
Serum *B. typhosus*	Serum dilutions	Type of aggluti-nation	2 hours	6 hours	24 hours	2 hours	6 hours	24 hours	2 hours	6 hours	24 hours
Rabbit 44	1 : 50	*s*	+ +	+ + ±	+ + +	(±)	±	+	+ + ±	+ + +	+ + +
(pure O	1 : 100		+ ±	+ + ±	+ + ±	(±)	(±)	±	+ + ±	+ + +	+ + +
serum)	1 : 200		+	+ +	+ + ±	–	(±)	(±)	+ +	+ + ±	+ + +
	1 : 500		+	+	+ +	–	–	(±)	+ ±	+ +	+ + ±
	1 : 1,000		(±)	±	+	–	–	–	±	+	+ ±
	1 : 2,000		–	(±)	±	–	–	–	–	(±)	±
	1 : 5,000		–	–	–	–	–	–	–	–	(±)
Rabbit 25	1 : 500	*l*	+ +	+ +	+ +	+ + ±	+ + ±	+ + ±	+ + ±	+ + ±	+ + ±
(pure H se-	1 : 1,000		+ ±	+ ±	+ +	+ + ±	+ + ±	+ + ±	+ + ±	+ + ±	+ + ±
rum obtained	1 : 2,000		±	±	+	+ +	+ +	+ +	+ ±	+ +	+ +
by absorption	1 : 5,000		–	(±)	±	±	+	+ ±	±	+	+ ±
with *B. typho-*	1 : 10,000		–	–	(±)	(±)	±	±	(±)	±	±
sus heated to	1 : 20,000		–	–	–	–	–	–	–	–	–
100° C.)											
	Saline control		–	–	–	–	–	–	–	–	–

Formalin = commercial formalin containing 40 % formaldehyde.

degree of reaction were unaltered. On the contrary, as we see in Table III, the sensitiveness of the H antigen was markedly weaker in the suspension preserved for 10 days in saline without formalin than in that with formalin (autolysis of the less resistant, labile H antigen being most likely the responsible factor).

But in the case of the small-flaking O agglutination, the destructive action

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of phenol and formalin was very distinctly demonstrated. Although the reactibility with the O agglutinin had not been completely lost, it was greatly reduced. The difference as compared with the fresh suspensions or with those preserved in saline only, was most striking during the first hours of observation. After 2 and 6 hours these two suspensions showed complete macroscopic agglutination in high dilutions, while phenol and formalin suspensions gave very weak agglutinations, only observable by the lens and in the lowest dilutions. In the overnight results the differences were usually much less distinct, but, as a rule, even here the reactions with phenol and formalin suspensions remained markedly weaker than those with the two control suspensions.

In numerous experiments it was established that this holds true equally for broth suspensions and for those from agar growths and for *B. typhosus* as well as for the *Salmonellas*.

It was also found that there was no difference in results whether the suspensions were weeks or months old or whether they were used on the day of their production.

This made it seem possible that the observed inhibition of the stabilotropic O agglutination was due not to an impairment of the O agglutinogen, but to that of the corresponding O agglutinin. The influence of phenol and formalin on the course of the labilotropic H and the stabilotropic O agglutination with the living organisms was accordingly investigated. In these experiments, Tables IV and V, the serum dilutions were prepared in parallel with saline, with saline + 0·25 per cent. phenol and with saline + 0·5 per cent. formalin respectively and living bacteria were finally added in one drop of saline suspension. Thus, the actual agglutination tubes contained the same amount of the respective disinfectants as in the previous agglutination tests with preserved suspensions in Tables II and III.

Table IV. *Action of phenol* (0·25 per cent.) *and formalin* (0·5 per cent.) *on the agglutination of a fresh suspension of living* B. typhosus.

Serum . *typhosus*	Serum dilutions	Type of aggluti-nation	Agglutination of a fresh suspension of living *B. typhosus* 901 in: Saline +0·25 % phenol, 2 hours	Saline +0·25 % phenol, 4 hours	Saline +0·25 % phenol, 20 hours	Saline +0·5 % formalin, 2 hours	Saline +0·5 % formalin, 4 hours	Saline +0·5 % formalin, 20 hours	Saline, 2 hours	Saline, 4 hours	Saline, 20 hours
)bit 24 (re O serum)	1:50	*s*	(±)	+	++±	(±)	±	++	+++	+++	+++
	1:100		(±)	±	++	(±)	(±)	+±	++±	+++	+++
	1:200		(±)	(±)	+±	–	(±)	±	+±	++	++±
	1:500		–	(±)	±	–	–	(±)	±	+	++
	1:1,000		–	–	(±)	–	–	–	(±)	±	+
	1:2,000		–	–	–	–	–	–	–	(±)	±
	1:5,000		–	–	–	–	–	–	–	–	–
)bit 25 (ab-)ed with *B.* *ıosus* 100° (pure H ım)	1:500	*l*	++±	++±	++±	++±	++±	++±	++±	++±	++±
	1:1,000		++	++±	++±	++	++±	++±	++	++±	++±
	1:2,000		+	+±	+±	+	+±	+±	+	+±	+±
	1:5,000		±	+	+	±	+	+	±	+	+
	1:10,000		(±)	±	±	(±)	±	±	(±)	±	±
	1:20,000		–	–	–	–	–	–	–	–	–
Saline control			–	–	–	–	–	–	–	–	–

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Table V. *Action of phenol* (0·25 per cent.), *formalin* (0·5 per cent.) *and alcohol* (1·0 per cent.) *on the agglutination of a fresh suspension of living* B. proteus HX 19.

Rabbit serum	Serum dilutions	Type of agglutination	Agglutination of a fresh suspension of living *B. proteus HX* 19 in: Saline +0·25 % phenol			Saline +0·5 % formalin			Saline +1·0 % alcohol			Saline		
			2 hours	6 hours	20 hours	2 hours	6 hours	20 hours	2 hours	6 hours	20 hours	2 hours	6 hours	20 hours
HX 19, 100° C. (pure O serum)	1 : 50	*s*	±	++	+++	(+)	+	+±	++±	+++	+++	++±	+++	+++
	1 : 100		(±)	+	++	(±)	±	+	++	++±	+++	++	++±	+++
	1 : 200		(±)	±	+	−	(±)	±	+	++±	+++	+	++±	+++
	1 : 500		−	(±)	±	−	(±)	±	±	+±	++	±	+±	++
	1 : 1,000		−	−	(±)	−	−	(±)	−	(±)	±	−	(±)	±
	1 : 2,000		−	−	−	−	−	−	−	−	(±)	−	−	(±)
	1 : 5,000		−	−	−	−	−	−	−	−	−	−	−	−
HX 2, 60° C. (pure H serum for the heterologous strain *HX* 19)	1 : 50	*l*	++±	++±	++±	++±	++±	++±	++±	++±	++±	++±	++±	++±
	1 : 100		++±	++±	++±	++±	++±	++±	++±	++±	++±	++±	++±	++±
	1 : 200		++	++	++	++	++	++	++	++	++	++	++	++
	1 : 500		±	+	+±	±	+	+±	±	+	+±	±	+	+±
	1 : 1,000		(±)	±	±	(±)	±	±	(±)	±	±	(±)	±	±
	1 : 2,000		−	(±)	(±)	−	(±)	(±)	−	(±)	(±)	−	(±)	(±)
	1 : 5,000		−	−	−	−	−	−	−	−	−	−	−	−
Saline control			−	−	−	−	−	−	−	−	−	−	−	−

Table VI. *The nature of the inhibition action of phenol and formalin as demonstrated by removing the inhibitive agents.*

Serum Rabbit 30 (*B. typhosus* 100° C.) (pure O serum)	Serum dilutions	Agglutination of a fresh suspension of living *B. typhosus* (strain No. 1) in: Saline +0·25 % phenol				Saline +0·5 % formalin				Saline			
Series I		2 hours	3 hours	6 hours	20 hours	2 hours	3 hours	6 hours	20 hours	2 hours	3 hours	6 hours	20 hours
Agglutination as usually performed	1 : 50	−	−	±	+	−	−	(±)	(±)	±	+±	++	++
	1 : 100	−	−	−	±	−	−	−	(±)	±	+	+	+±
	1 : 200	−	−	−	(±)	−	−	−	(±)	(±)	±	±	+
	1 : 500	−	−	−	−	−	−	−	−	−	−	−	(±)
	1 : 1000	−	−	−	−	−	−	−	−	−	−	−	−
Series II		Before washing	After washing			Before washing	After washing			Before washing	After washing		
After 2 hours all the tubes centrifuged, the sediments washed and resuspended in saline	1 : 50	−	+±	++	++	−	+±	++	++	±	+±	++	++
	1 : 100	−	+	+	+±	−	+	+	+±	±	+	+	+±
	1 : 200	−	±	±	+	−	±	±	+	(±)	±	±	+
	1 : 500	−	−	−	(±)	−	−	−	(±)	−	−	−	(±)
	1 : 1000	−	−	−	−	−	−	−	−	−	−	−	−

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The result, as demonstrated with *B. typhosus* and *B. proteus X* 19, was that under these conditions of experiment the O agglutination was also inhibited in the same selective way, while the H agglutination took its course without any disturbance, exactly as in the saline control.

The following experiment, Table VI, was arranged in order to investigate the nature of this impairment; the agglutination tests with a fresh suspension of living *B. typhosus* were set up in duplicate with serum diluted in saline, in saline + phenol and in saline + formalin. After 2 hours at 37° C. the first reading was done, yielding, of course, identical results in both series. Then all the tubes of Series I were left at room temperature while each tube of Series II was centrifuged; the sediments, after removal of the supernatant fluid, were once washed with saline, resuspended with saline to the original volume of 1 c.c. and were then allowed to stand at room temperature together with the control Series I. The subsequent readings after 3, 6 and 20 hours—indicating the time elapsed since the agglutination tubes had been put in the incubator —revealed a very striking difference. While in Series I the tubes containing phenol and formalin showed the complete or partial inhibition described in the previous experiments, the washed and resuspended bacteria in Series II were agglutinated in all the columns of the table exactly to the same degree.

Therefore, one might conclude, that the toxophore group of the O agglutinin, while not destroyed by the weak concentrations of disinfectants used, is, nevertheless, prevented by their presence from carrying out its function. The haptophore group, known from all the older works as much more resistant towards various physical and chemical influences, is completely unaltered by these weak concentrations; its function, the binding of the O immune body with the corresponding O antigen, takes place quantitatively, as is shown in Table VI by the equally strong agglutination in all the columns of Series II.

But further experiments made it clear that the nature of this inhibition phenomenon is much more complicated. If the usual suspension of the normal H culture of the organisms was replaced by their O culture the inhibition of the O agglutination was, as a rule, not demonstrable at all. In the case of *B. proteus X* strains it has been described by Weil and Felix (1917) that the reaction of the O agglutinin occurs much more slowly with the living O culture of these organisms than with the living H culture, although the degree of agglutination, as shown by the overnight reading, is, as a rule, higher with the O culture. The same is valid in the case of *B. typhosus* and the Salmonellas. In numerous experiments with the O culture of two strains of *B. enteritidis* Gärtner and of *B. proteus X* 19 and *X* 2 it was seen that no inhibition of this slowly progressing O agglutination occurred. When it occurred exceptionally it was found that the particular O culture was not pure, but contained a certain amount of H antigen. The inhibition of the O agglutination could, therefore, not be explained by the direct action of the disinfectants on the toxophore group of the O agglutinin; the presence of H antigen in the bacillary suspension was a necessary condition for the appearance of the phenomenon.

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It was also found that if the O agglutination took place in the absence of phenol and formalin, leading to the so-called second phase of the reaction—the formation of flakes—the agglutinated bacteria could not be afterwards disagglutinated by centrifugation and resuspension in saline containing 0·25 per cent. phenol and 0·5 per cent. formalin. It could be assumed, according to the views formerly held, that the resistance of immune bodies bound up with the antigen, is greater than that of the free immune bodies. But as far as resistance to heat is concerned, this has already been proved by Spät (1922) not to be true.

In the case of typhus fever the inhibitive action of phenol and formaldehyde on the agglutination with *B. proteus X* 19 was observed many years ago by Csepai (1917), Sachs (1917), Schiff (1917) and Schiff and Nathorf (1920). Further, they established the fact that this inhibition does not occur after the heating of the suspensions to 60° C. or to higher temperatures, and that it is involved in the agglutination of the patients' serum only but not in that of rabbit immune serum. As similar conditions were not known in typhoid fever and *B. typhosus*, these facts seemed to many workers, but mistakenly, to give further support to the almost universal view of the non-specific nature of the Weil-Felix reaction. In the case of the Salmonellas, Bruce White (1926) mentioned in passing that he avoided the preservation of suspensions in 0·1 per cent. formalin, which he had largely employed in earlier work, for the reason that the reactivity of certain essential factors appears to decrease under this treatment.

It may be mentioned that all our experiments were carried out with *B. typhosus*, *B. paratyphosus A and B*, *B. enteritidis* Gärtner and with several strains of *B. proteus X*, and that the results were invariably the same. In the latter species the susceptibility of the O agglutination was still more markedly shown—especially when sera from typhus patients were examined instead of rabbits' sera—while the resistance of the H agglutination was the same as in the case of *B. typhosus* and the Salmonellas.

In exactly the same way as with phenol and formaldehyde, these experiments were also carried out with alcohol, whose selective destructive action on the H antigen is already sufficiently well known (Weil and Felix, 1920). The alcohol did not in any instance show signs of inhibitive action (see Table V), not even when employed in concentrations as high as 5 per cent. alcohol in the actual agglutination test.

Discussion.

Whatever may be the explanation of this phenomenon of inhibition the facts described seem to be of considerable importance from the practical point of view. First of all, it must be stated that suspensions preserved with phenol or formalin are not adequate reagents for the practice of the qualitative serum analysis. It is true that as far as the labilotropic H agglutination is concerned their sensitiveness is equal to that of living cultures. But they are far inferior to the living cultures in the demonstration of the stabilotropic O agglutination.

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The above observations make it obvious why various workers were always unsuccessful in their numerous attempts to apply suspensions preserved in the usual way for agglutination tests with *B. proteus X* 19. For, as has been sufficiently proved, the blood serum of typhus patients contains exclusively O agglutinins, and, in addition, the susceptibility—as already mentioned—of the O agglutination towards phenol and formalin is in this special case even greater than in the case of *B. typhosus* and the Salmonellas. For this reason there is only one single "diagnosticum" suitable for diagnosis in typhus fever, namely, the alcohol suspension of *B. proteus X* 19 recommended by Bien and Sonntag (1917).

In the present state of our knowledge we can see one way only of eliminating the suspension of living organisms from the routine diagnosis of enteric fevers. It is the use of two differently preserved bacterial suspensions, namely, the usual suspensions, preserved with phenol or formalin, as a reagent to demonstrate the H agglutinin and an alcoholic suspension, similar to that used by Bien and Sonntag in the case of *B. proteus X* 19, as a reagent to demonstrate the O agglutinin. As a matter of fact, Schiff (1925) has already made such a suggestion.

It might be objected that the performance of the Widal reaction would in this way become unduly complicated. But this objection carries little weight, since the qualitative method renders superfluous the titration up to the end point of the reaction and only requires one single serum dilution for every organism used. Therefore, in using two such preserved suspensions, it would even be possible to reduce the amount of labour and material required by the quantitative methods hitherto in use.

But this matter does not only interest those who, like ourselves, are convinced that the qualitative method is in fact capable of yielding those advantages which have been claimed for it.

On the basis of our experience we may state with certainty, that the demonstration of the O agglutinins is also of great importance for the purposes of simple diagnosis of typhoid cases.

It is true that both agglutinins are formed in the majority of typhoid cases. But there may be great variations in the time of their appearance during the course of the disease and in many cases it is the O agglutinin which appears first and is, therefore, the decisive factor in the early diagnosis (Felix, 1924). But, what is of even greater importance is, that in a considerable percentage of typhoid cases the H agglutinin is totally absent during the whole course of the disease. Not less than 27 per cent. out of 521 cases of typhoid fever which we observed in Palestine during the years 1923–26 yielded a serum at some stage or other of the disease that contained only the O agglutinin. This figure varied considerably in different years of observation and also in relation to the locality from which the patients came. It is quite possible that epidemics in different countries vary in this respect. But the fact remains that one must count on the absence of the H agglutinin in a considerable percentage of all cases.

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In these cases suspensions preserved in the usual way, must, according to the previous statements, prove a failure whereas the living cultures will yield a positive Widal test. Such observations, referring unfavourably to Ficker's diagnosticum, were indeed made shortly after its introduction (Güttler, 1904; Vorwoort, 1905; Schrumpf, 1907; Goethlein, 1907, quoted from Paltauf, chapter on Agglutination in Kolle-Wassermann). As they were not very numerous they were accounted inexplicable and were neglected. The number of these observations would probably increase if the first examination of the blood were as a rule made in the earliest possible stage of the disease, say, before the end of the first week. It also seems probable that in a certain proportion of cases the negative Widal reaction found throughout the disease was really due to the effect of the preserved suspension used.

We must emphasise that we had no opportunity of employing Dreyer's technique. Our investigations were limited to testing preserved suspensions, as used on the Continent, which are always prepared with 0·5 per cent. phenol and 1·0 per cent. formalin. The objection can therefore be raised, that our conclusions are not valid for the employment of the most refined technique as developed by Dreyer, where the formalin employed is of a tenfold weaker concentration, 0·06 per cent. formalin in the actual agglutination test.

As far as the typhus agglutination with X 19 is concerned, it was found that the presence even of 0·05 per cent. formalin in the agglutination tubes causes the described inhibition of the O agglutination. This result is in agreement with a statement of Dr Gardner, from the Standards Laboratories, Oxford, that it was not possible to obtain a satisfactorily preserved suspension of X 19 even by the use of Dreyer's method. In the case of the enteric fevers it might be objected that the presence of this small quantity of formalin may not influence the less susceptible O agglutination at all, or only to a small extent. But an explicit statement in the above mentioned Report of the Committee upon Pathological Methods, 1920, p. 135, does not seem to speak in favour of this view. This statement is: "It is somewhat surprising to find that the phenomenon of group agglutination is far less conspicuous with this technique than with those previously used. The reason is not apparent, but the fact is attested by all who have worked with this method, and has been found advantageous in avoiding the confusion in diagnosis which group agglutination sometimes causes." Now, group agglutination in the enteric fevers is caused exclusively by O agglutinins (Weil and Felix, 1920); if it appears less markedly with Dreyer's technique, then the latter method also seems to exhibit the *described inhibition in O agglutination.*

The observations of Pijper (1923) in South Africa may serve as further evidence. It is true that the far-reaching conclusion of Pijper as to the unreliability of the Widal test is in no way justified. But the fact, underlying this conclusion, that out of 120 undoubted cases of typhoid fever as many as 34, or 28 per cent., yielded a negative Widal reaction, is well worthy of consideration. The apparent absence of H agglutinin in this series of Pijper recalls

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the fact mentioned above that 27 per cent. of our Palestine cases contained no H agglutinin but only O. In the light of our experiments a completely negative Widal might be quite intelligible as the technique used by Pijper resulted in a final concentration of 0·1–0·2 per cent. formalin in the actual agglutination test. But the original Oxford suspension, used by Pijper together with other control suspensions, yielded in these cases the same unsatisfactory results.

For several reasons standardisation according to Dreyer's principle seems even more desirable in the case of the proposed suspension preserved with alcohol than in the case of the formalinised suspension. As has been shown by Weil and Felix (1920) various strains of *B. typhosus* differ very much in the sensitiveness of their O antigen towards the O agglutinin. And it is just this agglutinin which is responsible for the agglutination obtained with the normal serum of human beings as well as of many species of animals (Schiff, 1922). But the details of this matter cannot be dealt with here.

Finally, it may be mentioned that the conclusions arrived at in the case of *B. proteus X*, *B. typhosus* and the Salmonellas, cannot, without further investigation, be held to apply to other species which do not possess the double type of antigen and antibody.

SUMMARY.

1. In *B. typhosus*, *B. paratyphosus A and B*, and *B. enteritidis* Gärtner, low concentrations of phenol and formaldehyde produce a definite inhibition of the O agglutination, while the H agglutination is unaltered—a fact already established many years ago in the case of *B. proteus X* 19.

2. Alcohol, even in high concentrations, has no such inhibitory effect.

3. Therefore, suspensions preserved in the usual way with phenol or formalin are not adequate reagents for the qualitative serum analysis in enteric fevers or for the usual diagnosis test in typhus fever.

4. Phenol and formalin suspensions are responsible for negative results of the Widal test in cases of enteric fevers which show only the O immune body in the serum; such cases form a considerable percentage of the total especially in typhoid fever.

5. As a routine measure in the diagnosis of enteric fevers, it is suggested that two preserved suspensions of each organism be used:

(1) an alcoholic suspension (like that of Bien and Sonntag in typhus fever) as a reagent for the O agglutinin, and

(2) the usual phenol or formalin suspension as a reagent for the H agglutinin.

6. The nature of the described phenomenon of inhibition is not, as yet, entirely clear.

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