SPECIFIC AND NON-SPECIFIC SERUM REACTIONS IN TYPHUS FEVER.

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CONTENTS.

Introduction.

In a previous paper (Felix and Rhodes, 1931) the occurrence of agglutinins for different serological types of *B. proteus* X in the serum of patients suffering from various forms of typhus and typhus-like diseases was discussed and illustrative data presented. The conclusion arrived at was that these diseases represent serological varieties of typhus and that antigenically their respective viruses correspond to serological types of *B. proteus* X.

In the present paper the nature of this agglutination reaction is discussed.

It is well known that in the past this problem was the subject of much controversy. On the one hand the X 19 reaction in typhus fever has been generally recognised as one of perfect "clinical" specificity and is now accepted as one of the most reliable serological tests used in routine diagnosis. On the other hand, the views held with regard to the nature of the reaction are still as controversial as they were ten years ago: in the opinion of most bacteriologists—at least as far as it is expressed in text-books—the reaction is non-specific in nature, unrelated directly with the typhus virus, while those who hold the opposite view represent a very small minority.

Numerous theories have been put forward with the view of proving the

hypothesis of the non-specificity of the Weil-Felix reaction (for references see Wolff, 1922; Wilson, 1929; Otto and Munter, 1930). The most favoured amongst them are (1) the paragglutination theory (Otto, 1919), (2) the theory which postulates peculiar and constant physico-chemical changes of the blood-serum of typhus patients (Epstein, 1919, and others) and (3) Wilson's (1920,1927) hypothesis according to which the X 19 reaction is one instance of "heterologous" agglutination of which other instances had been described by Wilson (1909, 1910) and others. It is not proposed to consider here these theories nor several others which have been suggested by other workers. In this paper one argument only will be discussed which has been most widely used in the course of this controversy and has served as the common basis of all the theories mentioned. It is the alleged fact that it is a peculiarity of the blood serum of typhus patients to develop agglutinins for a variety of bacteria and that *B. proteus* X 19 is only one out of a number of organisms which can be used for the detection of these agglutinins. On the Continent the conception has been formulated of a "polyagglutinating" capacity peculiar to the blood serum of typhus patients, while in this country the phenomenon is usually ascribed to the presence of heterologous agglutinins, so-called.

I. REVIEW OF EARLIER LITERATURE.

(*a*) *"Heterologous" agglutinins.*

The occurrence of "heterologous" agglutinins in the serum of patients and of immunised animals has been recorded since the early days of the study of the agglutination phenomenon (for references see Paltauf, 1904, 1913; Wilson, 1909, 1920). The term was introduced to separate the so-called group agglutination, which is due to common group antigens, from an agglutination reaction occurring between a heterologous serum and an organism which does not possess any demonstrable group antigen in common with the organism homologous to the serum. The differentiation between group agglutinins and heterologous agglutinins has been based on the result of the absorption test; the former are removed from the serum by absorption with the homologous organism while the latter are left intact.

Many of the earlier observations on heterologous agglutinins, especially those recorded in the case of human patients' serum, are probably due to an erroneous interpretation of results, caused by the imperfect knowledge of various sources of error which have been elucidated by subsequent investigations. Some of these pitfalls of the agglutination reaction are: the incidence in the normal serum of man and animals of agglutinins often in high titre, especially for *B. coli* and other intestinal bacteria; re-stimulation of persisting agglutinins, due to a previous infection or inoculation, under the non-specific stimulus of another infection or immunisation; non-specific serum agglutination of suspensions of partially "rough" organisms; various degrees of community of antigen amongst members of different species. However, other

observations clearly indicate that instances of significant rise and decline in the titre of heterologous agglutinins have been established beyond doubt.

Heterologous agglutinins in the serum of immunised animals have been demonstrated first by Posselt and Sagasser (1903) and also by Ballner and Sagasser (1904). In human patients' serum they have been carefully studied by Wilson (1909, 1910) in cases of cerebro-spinal fever and typhus fever. *B. coli* and *B. aquatilis alcaligenes*, an organism isolated from Belfast tap water and resembling the *B. faecalis alcaligenes*, were found to react significantly with the serum of patients suffering from cerebro-spinal fever. In one case the curves of agglutinin formation for the latter organism and for the meningococcus were compared thoroughly and were found to a certain extent parallel. The blood serum of typhus patients agglutinated several organisms isolated from these cases, viz. diplococci from the blood, *B. coli communis* from the urine and a variant of *B. coli communis* from the faeces, designated Bacillus U. From these observations Wilson reached the conclusion that heterologous agglutinins for various saprophytic organisms occur in the patients' serum in the course of these two diseases.

The origin of heterologous agglutinins and their relationship to the normal agglutinins have not yet been explained satisfactorily. It is not yet decided whether the rise and decline of heterologous agglutinins are due to the antigenic action of the infecting organism or to that of the saprophytic organism concerned, which is harboured by the particular individual and favoured by the conditions created by an intercurrent infection or immunisation. Another theory is that heterologous agglutinins are identical with normal agglutinins increased by a non-specific stimulus; and a further theory explains the phenomenon by assuming physical and chemical changes in the blood serum, altered "globulins" being held to be the responsible factors.

(*b*) *The "polyagglutinating" capacity of typhus serum.*

There is no doubt that the serum of some typhus patients, like some other sera, does agglutinate various bacteria in higher dilution than normal serum.

The term "polyagglutinating" capacity of typhus serum was introduced by Weltmann (1916) to denote the supposed peculiarity possessed by the serum of typhus patients of agglutinating various kinds of bacteria. This view had been formed because each of the numerous workers, who at some time or other had described a presumed causal organism of typhus fever, had also described an agglutination reaction in the attempt to prove its etiological significance. These results, however, could not be confirmed and the analysis of the data referring to these agglutination tests clearly indicated that they had been obtained by inaccurate methods (for references see Felix, 1918; Weil, 1920). Indeed, some of the authors themselves stated that their results were not satisfactory and could not carry weight. Nevertheless, undue importance was attached to their observations by subsequent workers (Rocha-Lima, 1919, and others) and the fact that for many years they were almost

generally accepted as valid evidence of the polyagglutinating property peculiar to typhus serum had a decisive effect on subsequent research on typhus fever. In a recent paper Weigl (1930) admitted this misleading effect on his own previous work (Weigl, 1923, 1924) and justly stated that nothing but a bad reputation had been preserved with regard to the numerous organisms which in the past had been brought into etiological relationship with typhus, mainly or partly on the basis of inadequate serological tests.

In addition to various organisms isolated from cases of typhus fever others have been described which are not related in such a way to the disease, but are still considered to be specifically agglutinated by the patients' serum. *Br. melitensis* may serve to illustrate how imperfect technique and imperfect judgment have combined in the formation of the opinion that a polyagglutinating capacity is peculiar to typhus serum and responsible also for the X 19 reaction. Nicolle and Comte (1910) published the following results of agglutination tests with *Br. melitensis* and the serum of typhus patients in Tunis (see Table I).

Table I. *Agglutination of* Br. melitensis *by the serum of sixty-eight typhus patients in Tunis* (*Nicolle and Comte*, 1910).

Titre of agglutination	No. of cases	%
1 : 10	12	17·64
1 : 20 till 1 : 50	18	26·47
1 : 50	14	22·5
1 : 100	1	
Total of agglutinating sera	45	66·61

They found that in 66 per cent. of cases an agglutinating action of the serum on *Br. melitensis* had been established, although they pointed out at the same time that in undulant fever a titre lower than 1 : 50 should not be taken as diagnostic of the disease. They recommended an agglutination test with *Br. melitensis* for routine diagnosis of typhus cases. Although the presumed peculiar action of typhus serum on *Br. melitensis* could not be confirmed by other workers (Negre and Raynaud, 1911; Rizutti and Scordo, 1912; Markl, 1913; Felix, 1918), and although it is known that in the blood of normal persons agglutinins may be present for this organism, sometimes up to a titre of 1 : 50 or 1 : 100, nevertheless the statement was repeated again and again in the literature on this subject that, according to Nicolle and Comte, typhus serum agglutinates *Br. melitensis* in 66 per cent. of cases (Rocha-Lima, 1919; Wolff, 1922; Otto and Munter, 1930, and others).

The non-specific re-stimulation by typhus fever of agglutinins for *B. typhosus* due to inoculation or previous enteric infection (Weil and Felix, 1916; Felix, 1917, 1929) was also considered, but mistakenly, to be peculiar to typhus serum.

In a previous paper (Felix, 1918) the "polyagglutinating" action of typhus and non-typhus sera on a number of "polyagglutinable" organisms, which, it was suggested, were specifically agglutinated by typhus serum, was compared

with the action of the same sera on *B. proteus* X 19. The non-specificity of the former was as evident as was the specificity of the latter reaction, since the polyagglutinable organisms reacted in a similar manner with both the typhus and the non-typhus sera while the X 19 did not. It was demonstrated in that paper and again emphasised by Weil (1920) that in comparative investigations of that kind it was indispensable to pay attention to the proper selection of adequate controls. It is obvious that in order to prove a supposed peculiar relationship between typhus serum and a polyagglutinable organism the latter ought also to be tested with sera from such other pathological conditions as are also known to cause an increased agglutinating power for various saprophytic organisms. Tuberculosis, syphilis, leprosy and other severe diseases are known to belong to this category. If, however, the controls are taken indiscriminately from cases of various diseases, including mild pathological conditions, surgical cases or even healthy individuals, then the polyagglutinating capacity developed in the course of typhus and other severe diseases may simulate specificity.

This essential condition of experiment was entirely neglected in the work concerning *B. pyocyaneus* Z 1 (Kreuscher, 1918; Neukirch and Kreuscher, 1919) and *B. agglutinabilis* U_2 (Wilson, 1927), the two organisms which have received much prominence in typhus literature. Wilson (1929) still holds the view that for the serological diagnosis of typhus fever these organisms "are often found almost equally serviceable" (p. 319) with the *B. proteus* X strains. Otto and Munter (1930) make the equally unwarranted statement that these strains of *B. pyocyaneus* and *B. coli* exhibit the same "outspoken specific affinity to the typhus patients' serum" (p. 1167) as *B. proteus* X strains do. With regard to *B. pyocyaneus* Z 1 the statements of the authors to whom they refer read as follows: "It has to be decided by further investigations and controls how far the phenomenon is strictly specific" (Kreuscher, 1918). And later: "It has been shown that the relationship between typhus sera and Z 1, which does not deserve the name specificity, is at least a peculiarity which might be taken into consideration in connection with the question as to the nature of the Weil-Felix reaction" (Neukirch and Kreuscher, 1919, p. 82).

It has been shown by Weil and Felix (1921) that rabbits infected with typhus virus invariably develop agglutinins for *B. proteus* X 19 but none for *B. pyocyaneus* Z 1 nor for other polyagglutinable organisms. The non-specificity of the agglutination reaction which Z 1 gives with the serum of some typhus patients has, therefore, been proved experimentally. Since *B. agglutinabilis* U_2 has not hitherto been tested in similar experiments and in view of the misleading statements previously mentioned a comparative investigation of the reactions which U_2 and X 19 give with human patients' sera was undertaken (see below).

(c) Weltmann's globulin test and the Wassermann reaction in typhus serum.

Weltmann (1916, 1917) described the phenomenon that in typhus the dilution (1 : 10) of the patients' serum with distilled water produces turbidity and suggested the use of this reaction for the diagnosis of typhus cases. The method was soon shown to be worthless since the test was not uniformly positive in typhus and was also met with in various other conditions. A similar phenomenon was known to occur with lues serum (Klausner, 1908) and is due, in both instances, to an alteration of the serum globulins (Epstein, 1919).

A transient positive Wassermann reaction in typhus was first described by Delta (1915), Papamarku (1915), Gotschlich, Schürmann and Bloch (1915) and later confirmed by Bauer (1921), Cruickshank (1927) and others. It does undoubtedly appear in a great proportion of cases in the course of the disease and becomes negative again during convalescence. It is known that the same phenomenon occurs in various infectious diseases, other than syphilis, such as malaria, leprosy, etc.

Both these non-specific serum reactions, though evidently not peculiar to typhus serum, were also brought into connection with the Weil-Felix reaction and it was suggested that all of them were due to one common factor—viz. to changes in the physico-chemical state of the serum. It is obvious that the supporters of this view entirely neglect the fact, established by those who have had the largest experience with typhus in various parts of the world, that non-specific reactions with X 19 do not occur in all those diseases which are known to produce a positive Wassermann reaction or an increase in the globulins of the blood serum. In typhus the independence of the result of the Wassermann reaction and of agglutination and complement fixation with X 19 has been shown by Reichenstein (1917) and the lack of correlation between the alteration of serum globulins and the titre of agglutination with X 19 is evident even from the data published by Epstein and Morawetz (1917).

II. COMPARATIVE OBSERVATIONS ON SERUM AGGLUTINATION WITH *B. PROTEUS* X 19 AND *B. AGGLUTINABILIS* U_2 (WILSON).

(a) Agglutination with normal and immune rabbit serum.

Prof. W. J. Wilson, Belfast, very kindly sent me a culture of his coliform strain *B. agglutinabilis* U_2. Its cultural characters were described by Wilson (1927) and in my hands it differed from the original description in one respect only. Wilson stated that in young cultures the bacilli were actively motile, while in my experience over a period of three years they were non-motile when grown on ordinary agar. Fletcher, Lesslar and Lewthwaite (1929) also described it as non-motile. Accordingly, no H agglutination has been observed with U_2, either in normal and immune sera from animals or in human sera. The agglutination reactions obtained with this organism and recorded in this paper were exclusively of the O type and they were compared only with the

O agglutination of X 19. It is known that only this type of agglutination is the decisive criterion in the diagnosis of typhus fever; further, it is established that normal agglutinins in human serum and in that of the customary small laboratory animals are O agglutinins (Rotky, 1921; Schiff, 1922). For these reasons no attention was paid throughout this investigation to H agglutinins for *B. proteus* X 19 which exceptionally are met with in human serum and still more rarely in small laboratory animals.

Since "roughness" of bacterial suspensions is one of the most important sources of error in agglutination tests much attention was directed to this point, and according to Arkwright's (1921) technique controls with saline solutions of increasing salt concentration (up to 6·8 per cent. NaCl) were included in each agglutination test. Both strains, U_2 and X 19, invariably gave stable suspensions indicating perfect "smoothness."

Wilson (1927) found in absorption tests with typhus serum that the agglutinins for U_2 and X 19 were separate and distinct. Nevertheless, it was carefully tested whether the two organisms do not share a common O antigen. Serum from twenty-five rabbits immunised with genuine and with variant strains of *B. proteus* X of all the known types (see Felix and Rhodes, 1931) and serum from five rabbits immunised with *B. agglutinabilis* U_2 were tested for cross-agglutination. In accordance with Wilson's finding no community of antigen could be established between the two organisms, although the sera used reacted up to high titres with their respective homologous organisms.

These tests, however, revealed the interesting fact that the serum of normal rabbits contains agglutinins for U_2 in much higher titre than for X 19. It has been shown by Weil and Felix (1921) that normal rabbit serum in dilution 1 : 10 very rarely reacts with either the H or O variants of X 19 and that agglutinins often cannot be detected even in a dilution 1 : 5.

Table II. *Agglutination of* B. agglutinabilis *U_2 and* B. proteus *X* 19 *by thirty-two sera of normal rabbits.*

Titre	No. of sera reacting with *B. agglutinabilis* U_2	No. of sera reacting with *B. proteus* X 19
<1 : 10	1	29
1 : 10	8	3
1 : 20	17	0
1 : 50	4	0
1 : 100	2	0
1 : 200	0	0
Total	32	32

In Table II the titre of normal agglutinins occurring in rabbit serum for *B. agglutinabilis* U_2 and *B. proteus* X 19 is shown. The difference is very striking.

(*b*) *Agglutination with normal and immune chicken serum.*

Friedberger, Zorn and Meissner (1922), using immune sera from chickens, succeeded in demonstrating community in the O antigen of the X strains and

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of *B. pyocyaneus* Z 1, the polyagglutinable organism previously mentioned. Four chickens were, therefore, immunised with the *Proteus* strains X 19, X_2, XK and a variant derived from X 19, respectively, and one chicken was immunised against *B. agglutinabilis* U_2. Sera with high O titres (up to 1 : 10,000) for the homologous organisms were obtained. No trace of overlapping agglutinins could, however, be disclosed between U_2 and the four strains of *B. proteus* X.

Again, the titration of the sera of the chickens, before immunisation was begun, showed a very great difference in the amount of normal agglutinins for U_2 and X 19.

Table III. *Agglutination with normal chicken serum.*

		Agglutination with strains		
		B. proteus X 19		*B. agglutinabilis*
Chicken no.	Serum dilution	H variant	O variant	U_2
1	1 : 25	+	+ ±	+ +
	1 : 50	−	±	+ ±
	1 : 100	−	−	±
	1 : 200	−	−	−
2	1 : 25	+	+ +	+ + +
	1 : 50	−	+	+ + +
	1 : 100	−	−	+
	1 : 200	−	−	−
3	1 : 25	+	+ +	+ + +
	1 : 50	−	±	+ + +
	1 : 100	−	−	+ + ±
	1 : 200	−	−	+
	1 : 500	−	−	−
4	1 : 25	±	+ ±	+ + +
	1 : 50	−	±	+ + +
	1 : 100	−	−	+ + +
	1 : 200	−	−	+ +
	1 : 500	−	−	±
5	1 : 25	+ +	+ + ±	+ + +
	1 : 50	±	+	+ + +
	1 : 100	−	±	+ + ±
	1 : 200	−	−	+
	1 : 500	−	−	−
Controls NaCl (%)	0, 85	−	−	−
	1, 7	−	−	−
	3, 4	−	−	−
	6, 8	−	−	−

Agglutination with fresh saline suspensions of living bacteria. Total volume 1 c.c. Reading after 20 hours; 2 hours' incubation at 37° C., then at room temperature.
+ + +, + +, +, ± = varying degrees of agglutination, estimated with the naked eye.
The degree of agglutination indicating the "titre" in all the other tables corresponds to the sign ±.

In Table III the results obtained with U_2 and with both the H and the O variant of X 19 are reproduced. That O agglutination with the O culture generally gives a higher titre than with the H culture has been shown by Weil and Felix (1917, 1921), the proportion in the titres reached being about 2 : 1. In the following experiments, however, the H culture only has been used, since almost all the data published on the Weil-Felix reaction have been obtained with the H variant of X 19.

The difference in the titre of normal agglutinins for *B. agglutinabilis* U_2 and for X 19 was as conspicuous with chicken sera as with rabbit sera.

(*c*) *Agglutination with normal guinea-pigs' serum.*

The guinea-pig is known for its poor capacity for producing immune bodies and most of the earlier workers also agree that guinea-pigs' serum is remarkably poor in normal agglutinins for various organisms. It is the animal placed at the bottom of the scale in which various animal species have been arranged according to the amount of normal agglutinins contained in their blood serum (Bürgi, 1907; Mamlok, 1909).

The very great difference in the titre of normal agglutination with U_2 and X 19, previously established with rabbit and chicken sera, was also found with the serum of normal guinea-pigs (see Table IV).

Table IV. *Agglutination of* B. agglutinabilis U_2 *and* B. proteus *X* 19 *by thirty sera of normal guinea-pigs.*

	No. of sera reacting with	
Titre	U_2	HX 19
<1 : 10	8	28
1 : 10	15	2
1 : 20	6	0
1 : 50	0	0
1 : 100	1	0
1 : 200	0	0
Total	30	30

(*d*) *Agglutination with normal human sera.*

Sera from out-patients which had been tested for the Wassermann reaction and had been found to give a negative test were used as "normal" human sera. Seventy-two samples of such serum were kindly supplied by Dr P. Fildes, London Hospital, and by Dr A. B. Rosher, Charing Cross Hospital. Only samples of serum were employed for agglutination tests which had not been inactivated. This precaution was taken because O agglutinins, to which type human normal agglutinins belong, generally possess remarkably low resistance to heat (Weil and Felix, 1917; Felix and Olitzki, 1929). Eight or ten days usually elapsed between the date of collecting the sera and that of testing them for normal agglutinins.

Simultaneously with the agglutination test Weltmann's globulin test was also performed with each serum. Two sets of serum dilutions 1 : 10 were put up in parallel, with normal saline and with distilled water, respectively, and were allowed to stand for 15 minutes at room temperature. Distinct turbidity occurring in the serum dilution with distilled water was then taken as indicating a positive reaction. None of the Wassermann negative sera gave a positive globulin test.

From Table V it is seen that in human serum, too, the titre of normal agglutinins for *B. agglutinabilis* U_2 exceeds that for *B. proteus* X 19 to a similar extent to that previously shown in various animal species. When the figures tabulated in Table V are compared with those specified in the previous tables

it will be found that, with regard to the amount of normal agglutinins contained in the blood serum, man holds a position intermediate between the guinea-pig

Table V. *Agglutination with normal human sera.*
(*Seventy-two Wassermann negative sera.*)

	No. of sera reacting with	
Titre	U_2	HX 19
<1 : 50	19	65
1 : 50	28	7
1 : 100	21	0
1 : 200	4	0
1 : 500	0	0
Total	72	72

and the rabbit on the one hand and the chicken on the other hand. This result is in keeping with the older work of Bürgi (1907) and others.

(*e*) *Agglutination with Wassermann positive sera from patients suffering from syphilis and leprosy.*

One hundred and thirty-eight samples of Wassermann positive lues serum were obtained from the two sources which supplied the Wassermann negative sera recorded in Table V. In both series the sera were manipulated in exactly the same way and in each particular experiment positive and negative sera were tested against the same bacterial suspensions side by side. The constancy of the sensitiveness to O agglutinins of both strains used was regularly controlled by standard rabbit immune sera and as an additional control positive and negative sera were repeatedly re-tested after having been stored in the ice box. The accurately quantitative character of the method in use was thus guaranteed throughout the long course of this investigation.

All the Wassermann positive sera were also tested by Weltmann's globulin test with the result that a positive reaction was obtained in a considerable proportion of cases. The degree of turbidity observed in the globulin test often corresponded to the intensity of the Wassermann reaction, a result which had been recorded by Dr Rosher in the case of fifty-seven sera supplied by him; however, exceptions to this rule were also noted, a fact well known from the observations made by earlier workers.

Table VI. *Agglutination with* 138 *Wassermann positive lues sera.*

	No. of sera reacting with	
Titre	U_2	HX 19
<1 : 50	30	126
1 : 50	38	12
1 : 100	41	0
1 : 200	21	0
1 : 500	6	0
1 : 750	1	0
1 : 1000	1	0
1 : 2000	0	0
Total	138	138

High titres of agglutination with U_2 were observed in numerous cases with these positive lues sera while in no instance did the titre for X 19 exceed that previously established with normal human serum. It is not proposed to compare the results obtained with normal and lues sera in terms of percentage, because of the comparatively small totals in both series. The figures in Tables V and VI clearly demonstrate that the agglutinating property of Wassermann positive lues sera is considerably increased for *B. agglutinabilis* U_2 while it does not exceed that of normal human serum with regard to *B. proteus* X 19. No constant relationship was, however, established between the intensity of the globulin and the Wassermann reactions on the one hand and the titre of agglutination with U_2 on the other hand.

Analogous results were obtained with the serum from patients suffering from leprosy (see Table VII). I owe these sera to the kind co-operation of Dr G. Stuart, Department of Health, Jerusalem, who also tested them for the Wassermann reaction and supplied the clinical data recorded in Table VII. The blood was drawn from 14 leper cases in Jerusalem on 22. ix. 30 and the sera, which had been neither inactivated by heat nor preserved by any disinfectant, were tested for their agglutinin content and by Weltmann's globulin test on 16. x. 30 in London. For reasons to be explained later the *Proteus* strains X_2 and XK and the *B. pyocyaneus* Z 1 have also been included in this experiment which is reproduced in detail.

Table VII. *Agglutination test with fourteen leper sera.*

Patient no.	Type of disease	Wassermann reaction: Citron's evaluation	Wassermann reaction: M.R.C. deviation in M.H.D.	Wassermann reaction: Weltmann's globulin test	Titre of agglutination with strains: U_2	HX 19	HX 2	HXK	Z 1
1	Nodular	+ +	3	±	*200*	0	0	0	100
2	Mixed	+ + + +	5	+	100	50	0	0	50
3	Mixed	+ +	3	−	100	0	0	0	50
4	Nervous	−	0	±	50	0	0	0	*200*
5	Nodular	+ + + +	5	±	*200*	0	50	50	100
6	Nodular	+ + + +	5	±	0	0	0	0	*200*
7	Mixed	−	0	−	50	50	0	0	100
8	Nodular	−	0	−	100	0	0	0	0
9	Nodular	+ + + +	5	+ + +	100	0	50	0	*200*
10	Nodular	+ + + +	5	±	100	0	0	0	50
11	Nervous	−	0	−	50	0	0	0	50
12	Mixed	+ +	3	+	*500*	0	0	50	100
13	Mixed	−	0	−	50	0	0	0	50
14	Mixed	+ + + +	5	+	*200*	0	0	0	50

Titre 0 = a negative result in dilution 1 : 50.

A more detailed analysis of the figures in Table VII is preferably reserved for a subsequent section of this paper. For the present two facts only may be emphasised: (i) U_2 is agglutinated distinctly higher by leper sera than by normal human sera (see Table V), while in the case of X 19 no difference whatever is detectable; (ii) high titres for U_2 are met with only in sera recorded as positive in both the Wassermann and the Weltmann tests (cases Nos. 1, 5,

12, 14); however, a strongly positive Wassermann or Weltmann reaction does not necessarily coincide with a high titre for U_2 (cases Nos. 2, 6, 9).

(f) Agglutination with sera from patients suffering from European classical typhus and from various typhus-like diseases.

In Table VIII are recorded the titres for U_2 and X 19 obtained with the serum from cases of classical European typhus and of those typhus-like diseases occurring in various parts of the world which, though not louse-borne, belong to the same serological variety, *i.e.* react with type X 19 of *B. proteus* X. The action of these sera on various types of *B. proteus* X has been described in a previous paper (Felix and Rhodes, 1931), where they have been specified in Tables I and VII under the numbers corresponding to those in Table VIII of the present paper. The American cases only, Nos. 8–17, had to be omitted in the previous paper, since their sera were preserved with glycerole which was found to cause non-specific agglutination of suspensions of the two strains HX_2 and OX_2. On *B. agglutinabilis* U_2 and on X 19 glycerole has no agglutinating effect; the glycerole sera could, therefore, be included in Table VIII.

As has been stated in the paper mentioned above rather long intervals usually elapsed between the date of collecting these exotic sera and that of testing them for their agglutinin content. This, however, does not vitiate the conclusions drawn from the examination of these sera, since the agglutinins for U_2 and X 19 considered here belong to the same type (O) and equally withstand the effect of ageing, whether the serum is preserved with or without the addition of glycerole (Wilson, 1927). None of the sera could, however, be tested for the Wassermann and the Weltmann reaction, for which purposes old sera are not suitable.

In Table VIII, as in all the tables in this paper, the degree of agglutination indicating the maximum titres corresponds to the sign ±, estimated with the naked eye, while in the previous paper (Felix and Rhodes, 1931) the sign + has been taken to mark the titre. This was necessary in order to increase the fineness of reading the low titre reactions between X 19 and normal agglutinins, which otherwise could not have been compared adequately with the much higher titres for U_2. Consequently, in Table V the figure stated for X 19 with normal agglutinins in human serum is higher by one serum dilution than that usually quoted, namely 1 : 50 instead of 1 : 25, and a similar difference is seen when the X 19 titres of the typhus sera in Table VIII are compared with the corresponding figures in the previous paper.

In Table VIII the titre O denotes a negative result in the dilution 1 : 200. Reactions with stronger serum dilutions had to be disregarded owing to the fact that they often occur with normal human serum (see Table V). According to the accepted rule even the titre of 1 : 200 ought to be considered as insignificant, since it is met with in four out of seventy-two normal sera (Table V).

From comparison of Tables V–VIII it becomes perfectly clear that the

positions held by the two organisms under examination differ profoundly from each other. The agglutination of *B. agglutinabilis* U_2, occurring with relatively high titres in normal human serum, is distinctly higher with the serum from patients suffering from syphilis or leprosy and still more so with typhus serum; it is, however, far from being a constant feature of typhus serum. On the other hand, the agglutination of *B. proteus* X 19 with its very much lower incidence and titres in normal individuals is not in the slightest degree affected by the syphilitic or leprous infection, but it is invariably very much enhanced by a typhus infection.

So far as *B. proteus* X 19 is concerned Tables V–VIII once more demonstrate that its reaction with typhus serum is, in the strict meaning of the word, "peculiar" to the typhus infection. With regard to *B. agglutinabilis* U_2, however, these tables clearly show, that its reaction with typhus sera is not

Table VIII. *Agglutination of* B. agglutinabilis U_2 *and* B. proteus *X* 19 *by sera from cases of typhus and typhus-like diseases.*

Typhus patients from		Titre of agglutination with strains		Typhus patients from		Titre of agglutination with strains	
United States of America	Case no.	U_2	HX 19	Poland	Case no.	U_2	HX 19
	1	0	500		1	500	5000
	2	0	1000		2	200	5000
	3	0	500		3	200	500
	4	1000	5000		4	500	10000
	5	0	2000		5	200	5000
	6	0	1000		6	200	500
	7	2000	5000		7	0	1000
	8	0	5000		8	0	2000
	9	0	200		9	200	2000
	10	1000	5000		10	500	5000
	11	200	1000	Ireland	1	2000	5000
	12	0	500	Australia	1	0	20000
	13	0	2000	Malaya	1	1000	5000
	14	2000	2000	(Group "W")	2	1000	2000
	15	200	5000		3	500	2000
	16	200	1000		4	0	5000
	17	0	2000				

Titre 0 = a negative result in dilution 1 : 200.

"peculiar" to this disease, since similar though somewhat lower reactions occur in cases of syphilis and leprosy.

It has been already mentioned that neither the Wassermann nor the Weltmann reaction could be attempted with the typhus sera, the samples having been either too old or preserved with glycerole. It has, therefore, not been established whether or not these two reactions were positive in those typhus sera which strongly agglutinated the *B. agglutinabilis* U_2. It seems probable that this coincidence obtains in typhus as well as in leprosy and syphilis. If this assumption were shown to be correct it would also offer an explanation of the fact that the incidence of high titre agglutinations with U_2 is markedly higher in typhus than in syphilis or leprosy. It has been emphasised by Weltmann (1916, 1917) and by Epstein (1919) that the alterations of serum globulins occurring as a result of syphilis and of typhus infection are

not identical. This conclusion was drawn from the fact that in the two conditions the globulin test requires different dilutions of serum with distilled water and different times of observation in order to yield optimum results. According to these workers it would appear that the increase in water-insoluble globulin is greater in typhus than in lues serum and this difference may account for the somewhat stronger agglutinating effect on U_2 by typhus sera.

An attempt was made to analyse the mechanism of the non-specific, heterologous agglutination of "polyagglutinable" organisms like U_2 by "polyagglutinating" sera such as are exemplified by syphilis, leper and typhus sera. This has no direct bearing on the typhus reaction with X 19, since this organism is not "polyagglutinable" and its specific relationship to the typhus virus has been established experimentally (Weil and Felix, 1921, and others). The following experiments might, however, contribute to the still imperfect knowledge of "heterologous" agglutination.

III. NON-SPECIFIC AGGLUTINATION BY GELATINE.

An interesting hypothesis was suggested long ago by Bürgi (1907) with the view of reconciling some apparently inconsistent facts established in the study of normal agglutination. On the one hand, data are available (see Gibson, 1930) showing that agglutinins for various organisms, simultaneously present in a normal serum, are capable of specific absorption in exactly the same way as are immune agglutinins due to specific sensitisation. On the other hand, it has been established by numerous workers that the content of normal agglutinins varies considerably with different animal species and that their normal sera are either generally rich or generally poor in agglutinins for various kinds of bacteria. The latter fact was considered by Bürgi (1907) as highly suggestive of a non-specific factor being involved in the phenomenon. His theory, calculated to reconcile the specific and non-specific features of normal agglutination, assumes a combined action of two factors, analogous to the two stages of the agglutination phenomenon, viz. specific sensitisation by the agglutinin and non-specific flocculation by electrolytes. Accordingly, specific agglutinins for various kinds of bacteria, present in small amounts in normal sera, are held responsible for the first stage of the phenomenon, the titre of the reaction, however, being determined by the non-specific effect of colloids present in the serum of different animals in varying amounts and in different physico-chemical conditions.

Now, marked physico-chemical changes in the serum proteins have been established as a result of pathological conditions like typhus, syphilis, leprosy, which give rise to the development in the patient's serum of polyagglutinating properties. It seemed, therefore, advisable to analyse the latter phenomenon on the lines of Bürgi's theory.

Gelatine was selected as the colloid to be tested since there exists no method of isolating serum globulins free from serum agglutinins. Moreover, the

agglutinating effect of gelatine on bacterial suspensions had already been studied by Weil (1904), who concluded that the mechanism of this non-specific agglutination tallies with that of specific serum agglutination.

Table IX. *Agglutination by gelatine.*

% gelatine in normal saline	Agglutination with strains: *B. proteus* X 19 H variant	*B. proteus* X 19 O variant	*B. proteus* XK H variant	*B. proteus* XK O variant	*B. agglutinabilis* U_2	*B. pyocyaneus* Z 1	*B. typhosus* No. 901 H variant	*B. typhosus* No. 901 O variant	*B. paratyphosus* B
3, 0	+H	–	+ +H	(±) O	+O	+ ±O	+ + +H+O	±O	+ +H+O
1, 0	–	–	(±)	–	–	(±)	+ ±H	(±)	(±) H
0, 5	–	–	–	–	–	–	–	–	–
0, 1	–	–	–	–	–	–	–	–	–

3 per cent. gelatine in normal saline, adjusted to *p*H 7·4, sterilised at 100° C., prepared in bulk and further dilutions made with saline.
Fresh saline suspensions of living bacteria. Total volume 1 c.c.
Reading after 20 hours' incubation at 50° C.
± = last degree of agglutination estimated with the naked eye.
(±) = traces, estimated by means of a magnifying lens.

In Table IX the two polyagglutinable organisms U_2 and Z 1 are compared with H and O variants of *B. proteus* X and of some Salmonella types with regard to their sensitiveness to the action of gelatine. It is interesting to note that the colloid acts on both the H and the O antigenic components, leading to typical floccular and granular agglutination. As in specific serum agglutination so here also the titre of H is higher than that of O agglutination. The sensitiveness of the O reaction with the polyagglutinable strains U_2 and Z 1 is, however, not much more marked than that with the other organisms, which do not belong to this category.

Weil (1904) also described a summation effect of the action of gelatine and agglutinating serum. Minute amounts of both substances, incapable of causing visible agglutination when acting separately on the bacterial suspension, produced a distinct effect by combined action. Bacteria which were and were not polyagglutinable were, therefore, subjected to similar tests (Table X).

Table X. *Combined action of gelatine and specific immune agglutinins.*

Agglutination of homologous rabbit O immune sera with strains

B. proteus OX 19 Serum dilution	In saline	In saline +1 % gelatine	*B. typhosus* O 901 Serum dilution	In saline	In saline +0·5 % gelatine	*B. agglutinabilis* U_2 Serum dilution	In saline	In saline +1 % gelatine	*B. pyocyaneus* Z 1 Serum dilution	In saline	In salin +1 % gelatin
1 : 5000	+	+ + ±	1 : 20000	(±)	+	1 : 5000	+ ±	+ + +	1 : 2000	+	+ + +
1 : 10000	(±)	+ ±	1 : 50000	–	±	1 : 10000	±	+ +	1 : 5000	±	+ +
1 : 20000	–	+	1 : 75000	–	(±)	1 : 20000	–	+	1 : 10000	–	+
1 : 50000	–	–	1 : 100000	–	–	1 : 50000	–	(±)	1 : 20000	–	±
									1 : 50000	–	(±)
Controls	–	–	Controls	–	–	Controls	–	–	Controls	–	(±)

Technique, see Table IX.

It is seen from Table X that the summation effect described by Weil was produced in every instance. No difference between the various organisms could, however, be established as regards their sensitiveness to the combined specific and non-specific action.

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IV. NON-SPECIFIC STIMULATION OF AGGLUTINATING CAPACITY BY HETEROLOGOUS IMMUNISATION.

Although the experiments with gelatine had failed to reveal any marked difference in sensitiveness to non-specific agglutination between polyagglutinable and non-polyagglutinable bacteria, it was thought possible that other colloids, like serum proteins, might in this respect act differently. Serum from horses undergoing hyper-immunisation with unrelated bacteria (certain anaerobes) was, therefore, tested for heterologous agglutinins, since changes in the quantity and quality of serum globulins are well known in that condition.

Table XI. *Agglutination with serum of horses before and after immunisation with various types of anaerobes.*

			Titre of agglutination with strains						
			B. proteus X 19		*B. proteus* XK		*B. agglutinabilis*	*B. typhosus* No. 901	
No.	Horse serum	Homologous to serum	H variant	O variant	H variant	O variant	U_2	H variant	O variant
1	Before immunisation	0	0	100	0	100	500	100	200
	After immunisation with *B. tetani*	500	0	100	0	100	2000	100	200
2	Before immunisation	0	0	100	100	200	100	100	200
	After immunisation with *Vibrion septique*	500	0	100	100	200	500	100	200
3	Before immunisation	0	100	200	100	200	500	200	500
	After immunisation with *B. welchii*	1000	100	200	100	200	2000	200	500

Titre 0 = a negative result in dilution 1 : 100.
Horses immunised by intravenous injections of washed bacilli heated to 100° C. for two hours.

As is seen from Table XI, the polyagglutinable strain U_2 differs markedly from the other organisms tested at the same time, since it is the only one agglutinated by distinctly higher dilutions of the sera obtained after immunisation than by the corresponding normal sera. Further, this table shows that in horse serum, as in that of all the other animals tested, the titre of normal agglutination is higher for U_2 than for X 19 as well as for the Kingsbury type of *B. proteus* X (XK) and for a strain of *B. typhosus* (No. 901) which is known for its particularly high sensitiveness to O agglutinins. Weltmann's globulin test was negative with the sera taken before the commencement of the immunisation and was strongly positive with the immune sera.

V. ANTIGENIC RELATIONSHIP BETWEEN *B. AGGLUTINABILIS* U_2 AND *B. COLI COMMUNIS*.

The high incidence of normal agglutinins for *B. agglutinabilis* U_2, established with the serum of all the species tested, made it appear desirable to ascertain whether the O antigen of this particular strain was shared by other members of the *B. coli* group to an extent sufficient to account for the wide distribution of the corresponding antibody. Three high titre sera from rabbits immunised with *B. agglutinabilis* U_2 were tested against twenty-three strains of *B. coli* from the National Collection of Type Cultures. Twelve strains had to be

discarded owing to their "rough" condition; with the remaining strains the following results were obtained:

Four strains gave marked group agglutination with each of the three immune sera.

Two strains weakly reacted to two immune sera.

Five strains gave entirely negative reactions.

The six strains which did react to the immune sera originated from different localities and included Escherich's original strain of *B. coli communis*. This result seems to justify the conclusion to be drawn that the high incidence of normal agglutinins for U_2 is due to community of antigen between this particular strain and numerous other members of the *B. coli* group.

VI. Discussion.

The observations recorded in this paper traverse those published by Wilson (1927). In my opinion the discordance is due to Wilson's failure to pay due attention to a sufficient number of adequately selected controls. The total number of non-typhus sera tested by Wilson against *B. agglutinabilis* U_2 was forty-three and they were specified as follows:

Thirty-five sera were sent in for the Wassermann test.

Five sera were sent in for the Widal test.

Two sera from cases of scarlet fever.

One serum from a case of tuberculosis.

The habit of using as controls in agglutination tests sera which have been sent in for a Wassermann test, without knowing whether they give a negative or positive Wassermann reaction, is to be strongly deprecated because it is bound to lead to fallacious conclusions. Firstly, the majority of such sera generally give a negative Wassermann reaction and ought to be considered as "normal" and it has been emphasised in a previous section (p. 386) why normal sera cannot serve as adequate controls (Felix, 1918). Secondly, these sera are usually taken from the inactivated samples, left from the Wassermann test, and in doing so a very serious source of error is introduced since O agglutinins possess very low resistance to heat (Weil and Felix, 1917). Moreover, the globulin content of the serum markedly influences the heat destruction rate of agglutinins (Felix and Olitzki, 1929). Thus the usual procedure of heating the undiluted serum at 55° C. or 56° C. for $\frac{1}{2}$ or 1 hour causes a considerable loss in the titre of O agglutinins and this loss is enhanced by the increase of globulins which generally coincides with a positive Wassermann reaction. It is obvious, therefore, that inactivated sera are useless in comparative agglutination tests.

Fletcher, Lesslar and Lewthwaite (1929) confirmed Wilson's results. They made control tests with *B. agglutinabilis* U_2 and the blood of 478 persons who were not suffering from typhus. The description of the controls given by these workers reads as follows: "Most of these controls were hospital patients whose

blood had been sent to the laboratory for Wassermann or agglutination tests." Hence, their conclusion is invalidated for the same reason as that of Wilson.

It has already been stated by Wilson (1927) that typhus sera from Mexico failed to agglutinate U_2 while reacting with X 19. From Table VIII it is seen that in my experience too the incidence of increased agglutinins for U_2 was distinctly lower in cases of endemic typhus of the United States of America than in those of classical typhus from Poland. The viruses of Mexican Tabardillo and of endemic typhus of the United States share certain properties which distinguish them from European typhus virus (Mooser, 1928, 1929; Maxcy, 1929 *a*, *b*, *c*) and this is also reflected by differences in the reaction of patients' sera to various types of *B. proteus* X (Spencer and Maxcy, 1930; Felix and Rhodes, 1931). From an observation published by Havens (1927), who has had large experience with endemic typhus of the United States of America, a relevant inference can be drawn. Havens failed to confirm with American typhus sera that extreme heat-lability of X 19 agglutinins which numerous workers have established with patients' serum from European typhus (for references see Otto and Munter, 1930; Havens, 1927). In the light of our present knowledge the particular heat-lability of O agglutinins in typhus patients' serum, which markedly exceeds that of agglutinins produced by typhus virus in the rabbit, is most likely due to the globulin increase accompanying typhus infection in man (Felix and Olitzki, 1929). If, according to Havens, this heat-lability is not often met with in American typhus sera, absence of globulin changes is to be suspected as the most likely reason for this additional peculiarity of the American variety of typhus. This would account for Wilson's statement that Mexican typhus sera do not agglutinate U_2, an observation which accords with the results shown in Table VIII.

The same explanation suggests itself for the difference in reaction to *B. agglutinabilis* U_2 established by Fletcher, Lesslar and Lewthwaite (1929) of the two serological varieties of tropical typhus occurring in the Federated Malay States. They found the agglutination reaction with U_2 positive in the urban "W" form of tropical typhus, which corresponds to the X 19 type of *B. proteus* X, but negative with the rural "K" form of the disease, which is related to the Kingsbury type of *B. proteus* X. I have failed to confirm the conclusion arrived at by these workers, that the agglutination reaction with U_2 affords an additional distinction between the two forms of tropical typhus. This reaction is neither uniformly positive with the "W" form (see Table VIII) nor is it invariably negative with the "K" form of typhus. However, the incidence of agglutinins for U_2 is distinctly higher in the variety of tropical typhus which corresponds to type X 19 than in the "K" form of that disease or in tsutsugamushi, which two latter varieties of typhus react with the Kingsbury type of *B. proteus* X. It is suggested that the explanation for this difference is the same as in the case of American and European typhus.

From the analysis of the results recorded in Table VII it would appear that globulin increase is a necessary concomitant of increased heterologous

agglutination. However, the reverse of this relationship does not obtain. While the highest titres for U_2 and Z 1 invariably coincide with a positive globulin test, the latter is not always accompanied by a high agglutination titre for these two polyagglutinable organisms. It is further seen from Table VII that none of the four highest titres for U_2 coincides with any of the three highest titres for Z 1. Consequently the augmentation of the globulins cannot be the sole factor determining the increase in heterologous agglutination. It would appear that in order to produce that effect it is necessary for the increase of the globulins to occur in a serum endowed with a relatively large amount of normal agglutinins. It has been shown by Weil (1904) and is again demonstrated in Table X that summation effects can be obtained with a colloid and a specific immune agglutinin, when each is employed in concentrations incapable of producing *per se* a visible effect. The assumption appears to be justifiable that such a colloid will also enhance the action of preformed normal agglutinins.

It is not suggested that this is the sole mechanism involved in increased heterologous agglutination. However, in instances of polyagglutination like those of *B. agglutinabilis* U_2 and *B. pyocyaneus* Z 1, the combined action of abundant normal agglutinins and augmented serum colloids may well account for the phenomenon.

An abundance of agglutinins is found in the normal serum of man and animals for organisms like diphtheroids, cocci, *B. coli*, *B. faecalis alcaligenes*, etc., which are living as saprophytes in the human and the animal body. Indeed, the polyagglutinable organisms described at various times are almost exclusively drawn from that category and *B. agglutinabilis* U_2 is one of its typical representatives. In the case of *B. proteus* X 19, however, it cannot be shown that it is susceptible to the action of polyagglutinating sera. The fact that this organism reacts only with the serum of typhus patients cannot, therefore, be explained by the action of heterologous agglutinins.

Summary.

1. In all species of animals tested—rabbit, guinea-pig, chicken and horse—normal agglutinins for *B. agglutinabilis* U_2 are far more abundant than those for *B. proteus* X 19.

2. Human serum reacts in the same manner. Wassermann negative sera have been employed as normal sera. Their reaction in Weltmann's globulin test was also negative.

3. Wassermann positive sera from cases of syphilis and sera from lepers give distinctly higher agglutination reactions with U_2 than normal human sera. On X 19, however, those sera do not exercise any increased agglutinating action.

4. Similar though somewhat stronger reactions with U_2 are obtained with typhus sera. However, this reaction is far from being uniformly positive in typhus, while this is the case with the X 19 reaction.

5. The discordance between these results and those published by Wilson and by Fletcher, Lesslar and Lewthwaite is due to the failure by these workers to employ controls of suitable type.

6. In syphilis and leprous sera high titres of agglutination with U_2 coincide with globulin-increase as indicated by the Weltmann reaction.

7. The summation effect of the non-specific agglutination by gelatine and the action of specific immune agglutinins, described by Weil, is again demonstrated.

8. The combined action of an abundance of preformed normal agglutinins and of augmented serum colloids is suggested as one of the factors involved in increased heterologous agglutination (polyagglutination).

9. *B. agglutinabilis* U_2 is a typical representative of "polyagglutinable" organisms; *B. proteus* X 19, however, is not liable to the action of "polyagglutinating" sera.

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