THE TITRATION OF THERAPEUTIC ANTI-TYPHOID SERUM

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INTRODUCTION

AN account has been given in a previous paper of the immunizing procedures now employed in the preparation of anti-typhoid serum in the horse for therapeutic use in man (Felix & Petrie, 1938). The object of the present communication is to describe the methods used for the titration of the serum.

The efficacy of anti-typhoid serum, so far as the results of clinical trials permit a judgement, depends upon the presence in it of two essential protective substances, namely, the "O" and the "Vi" antibody. It follows that the titration of the serum is based on the separate quantitative estimation of each of these two components. During the past four years *in vitro* and *in vivo* methods of estimating the Vi and O antibody in therapeutic anti-typhoid sera have been used in routine and experimental work. While the tests for the determination of the O antibody are comparatively simple, those for the Vi antibody present technical difficulties which can be overcome only by experience and by due attention to a number of details that are discussed in this paper.

THE TITRES OF Vi AND O AGGLUTINATION IN RELATION TO THE
BIOLOGICAL ACTIVITIES OF THE Vi AND THE O ANTIBODY

The following facts have been established from previous experiments with anti-typhoid sera from the rabbit which contained Vi, O and H antibodies (Felix & Pitt, 1934*b*, 1935; Felix & Bhatnagar, 1935):

(1) The Vi titres of the sera, as estimated by the agglutination reaction, run, on the whole, parallel with their protective values as determined by tests in mice which received a test dose of living virulent typhoid bacilli.

(2) The O titres of the sera, as estimated by the agglutination reaction, run, on the whole, parallel with their protective values as determined by tests in mice which received a lethal dose of a suspension of heat-killed virulent typhoid bacilli.

(3) The ratio of the value of the serum in the protection test with the living bacteria to its Vi agglutinin titre is approximately constant for sera from rabbits which have been immunized with the "natural" Vi antigen as contained in the living bacterial cells. In this respect there is no difference between the Vi antigen from smooth virulent strains and that which is contained in rough avirulent strains.

(4) The ratio is quite different, in fact many times smaller, for sera from rabbits which have been immunized with formolized suspensions or extracts from Vi strains. The reason for this is that such sera contain a modified Vi antibody which is markedly deficient in its protective and phagocytosis-promoting functions, although its agglutinating activity in no way differs from that of the Vi antibody produced in response to immunization with the "natural" Vi antigen.

These findings have been amply corroborated by subsequent experience. Comparative titrations of immune sera from the rabbit and the horse invariably demonstrate the close correlation that exists between the results obtained in the two sets of *in vitro* and *in vivo* tests: on the one hand, between Vi agglutination and the mouse-protection test with the living bacteria, and on the other hand, between O agglutination and the mouse test for the neutralization of the toxic action of the killed bacteria.

It is admittedly very difficult to assess the relative importance of the antibacterial and the antitoxic mechanism in immunity to infection with bacteria which do not produce a typical "exotoxin". While the antibodies to exotoxins do not produce any direct antibacterial effect, the antibodies to the "endotoxins" exert both an antitoxic and an antibacterial action.

The typhoid bacillus is devoid of a typical exotoxin and the two antigenic constituents known as the O and the Vi antigen are at present recognized as the essential elements in the pathogenic and immunogenic characters of this organism. It is still, however, a matter for speculation what the exact nature of the mechanism is by means of which these two factors combine to confer upon the typhoid bacillus the highest degree of pathogenicity of which this organism is capable. Consequently the exact mode of action of the two corresponding antibodies is, as yet, little understood. The scope of the present communication is confined to the practical problem of the quantitative estimation of the two antibodies and does not permit of a discussion of this important question; the problem has been specially investigated by Ørskov & Kauffmann (1936), who believe the Vi antigen in the living bacteria to be some peculiar kind of toxin.

The sharp distinction between the activities of the Vi and the O antibody in mouse-protection tests is revealed only when strains are used that possess maximum virulence, are particularly rich in the Vi antigen, and manifest the highest degree of resistance to the O antibody. Most laboratory strains, however, contain only a moderate amount of the Vi antigen and consequently are less O resistant. When such strains are examined in bactericidal or phagocytic tests with O immune sera, a powerful sensitizing effect of the O antibody is readily demonstrable. Pure O immune sera, containing no Vi antibody, are therefore capable of exerting a strong protective action in the mouse-protection test when such cultures are employed for the test dose. If, however, the animals receive a test dose prepared from a strain of the highly O resistant type, the sensitizing action of the O antibody is feeble and is masked by the powerful phagocytosis-promoting activity of the Vi antibody (Felix & Bhatnagar, 1935). The contribution by the O antibody towards the sum total of protective action of a serum which possesses both the O and the Vi antibody appears to be under these conditions almost negligible; this accounts for the correlation between the protective action and the Vi agglutination titre of the serum.

In contrast to this result the O antibody plays the decisive role in the mouse test for the neutralization of the toxic action of killed typhoid bacilli.

It is not suggested that the O antigen is the sole factor associated with the toxic action of killed or living typhoid bacilli. Other antigenic and possibly also non-antigenic constituents of the bacterial cell may also be concerned in toxigenicity. But it is the O antibody alone of the three antibodies so far known to be present in anti-typhoid serum that shows a definite and constant relationship to the potency of the serum in neutralizing the "endotoxin" that is present in a suspension of the killed bacteria.

THE PROVISIONAL STANDARD SERUM USED IN THE TESTS

It was recognized early in this work that, whatever the methods employed, a reliable determination of the two antibodies could be effected only by the use of a standard serum, which was tested simultaneously whenever a determination of one of the two antibodies was made. The provisional standard serum was taken in February 1935 from the blood of the first horse that had been immunized with living bacilli of the rough avirulent Vi strain Ty 441. R5 (Felix & Pitt, 1935); this serum had a content of Vi and O antibodies which, at that time, was considered to be adequate. From the natural, unconcentrated serum, which did not contain any preservative, Dr P. Hartley, Director of Biological Standards, National Institute for Medical Research, very kindly prepared a considerable quantity of a dry stable standard serum, according to the method used for the preparation of standards for other therapeutic immune sera (Hartley, 1936).

Another portion of the original serum was kept in the liquid form without the addition of any preservative in sealed ampoules in the cold room, and its potency was compared from time to time with that of the dry standard. It was found that the process of drying the serum had not appreciably diminished its potency in the various *in vitro* and *in vivo* tests to which it was submitted and further examinations showed that no significant loss in the potency of the liquid serum was discernible after storage for periods up to three years. During that time the liquid serum could therefore be used as a laboratory standard for the routine titration of anti-typhoid sera. It was thus possible to economize the dry preparation, which was only occasionally used for checking the titres obtained with the liquid serum.

The reason why the potency of the liquid serum remained undiminished during such a long period appears to be the complete absence from the serum of any preservative whatsoever. Previous experience with various O and H immune sera had indicated that the titres of these antibodies were remarkably well maintained for periods up to five years, provided that the liquid serum was entirely free from any preservative.

THE STRAINS OF *BACTERIUM TYPHOSUM* USED IN THE TESTS

The provisional standard serum forms the basis of the measurement of both the Vi and the O antibody in all the tests employed in the titration of anti-typhoid sera. Nevertheless it is a matter of the greatest importance to use in

these tests only cultures from carefully selected strains, which possess certain essential characters and are known to retain them for long periods while the cultures are maintained in a relatively simple manner.

The necessity for using in the mouse-protection test with the living bacilli only cultures of maximum virulence and resistance to O antibody has been emphasized already in a previous section. Further, maximal O inagglutinability is the indispensable characteristic of any smooth culture of *Bact. typhosum* that is to serve as the reagent for the estimation of the Vi antibody by the agglutination test. The O agglutinin titres of therapeutic anti-typhoid sera of adequate potency are invariably much higher than the Vi agglutinin titres, the ratio usually being about 50:1 for the present batches of serum from the Serum Department of the Lister Institute (Felix & Petrie, 1938). If the Vi agglutination test is to be accepted as a workable method of titrating the Vi antibody the titres must be estimated by utilizing the unabsorbed sera under test, that is to say, despite the presence of O antibody of high titre.

Papers recently published by Pijper & Crocker (1937), Grasset & Lewin (1937), and others, and personal communications received from numerous workers illustrate the widespread belief that the determination of the Vi agglutinin titre of a serum which also contains O and H agglutinins can be carried out only after the complete removal of these antibodies by absorption. This point of view is, however, erroneous.

It is true that the technique of the Vi agglutination reaction is not so simple as those employed for H and O agglutination. The difficulties met with are due not only to the great variability in the content of the Vi antigen of the cultures but also to the fact that the end-point of the Vi agglutination, that is, the titre of the serum under test, is correlated in a peculiar manner with the amount of Vi antigen present in the test culture. The more Vi antigen a culture contains the lower is the titre of the Vi agglutination reaction obtained with the culture, and this rule is valid irrespective of whether an unabsorbed or an absorbed serum is being examined (Felix & Pitt, 1934*b*; Felix *et al.* 1934). This inverse relation, which is inherent in the test, was one of the reasons that led to the adoption, early in the work, of the dry standard serum, which proved to be the sole trustworthy basis for standardizing the sensitiveness of the Vi strains employed in the estimation of the Vi agglutinin.

The technique of the agglutination test, when using the so-called pure Vi serum, from which the accompanying O and H antibodies have been removed by absorption with the strain H 901, a strain that is devoid of Vi antigen, is wholly impracticable for routine purposes. The procedure is much too complicated to be applied to the routine examination of successive serum samples from horses undergoing immunization. Moreover, the treatment of the serum with the enormous number of bacillary bodies required for the complete absorption of the high-titre O and H antibodies, is bound to introduce various factors that would reduce the accuracy of the titration to such an extent as to render it useless as a quantitative test (Felix & Pitt, 1936; Detre, 1937).

Fortunately it is possible to estimate the Vi antibody titre by direct agglutination tests with unabsorbed sera, which also contain H and O agglutinins. The requisite conditions, which should be strictly observed, are:

- (a) the selection of strains known to maintain their Vi antigen content almost uniformly, so long as the cultures are properly treated;
- (b) standard conditions of maintenance of the cultures;
- (c) the inclusion in each agglutination test of controls with pure Vi, O and H sera of known titres.

The following table gives the list of strains of *Bact. typhosum* which have been employed for the titration of anti-typhoid sera during the past four years.

Table I. *Giving details of strains of Bact. typhosum used in the titration of anti-typhoid sera*

Strains	Year of isolation	Antigens present in the strains			Employed for
Ty 2	1918	Vi	O	H	Test dose in mouse-protection test with living bacilli
Watson	1932	Vi	O	H	Estimation of Vi agglutinin titre
Ty 6S	1936	Vi		H	
		(small amount)			
O 901	1925		O		Estimation of O agglutinin titre
					Test dose in mouse test for neutralization of the toxic action of killed bacilli
H 901	1918		O	H	Absorption of H and O agglutinins in the preparation of "pure" Vi serum

All the strains specified in Table I have been described in earlier work. The strain Ty 2 possesses the maximum virulence and O inagglutinability of which cultures of *Bact. typhosum* are capable when grown on ordinary media. Moreover, it is superior to other strains in maintaining these characters. Numerous authors, working in various parts of the world, have described strains of *Bact. typhosum* which, at the time of their isolation, possessed maximal virulence and maximal O inagglutinability. However, in most instances these properties are gradually lost on prolonged subculture on plain agar. They can be restored either by passage through the mouse, in the manner described by Perry *et al.* (1933) as the method of "rejuvenation" of the Rawlings strain, or by growing the cultures on agar containing ascitic fluid or on egg medium (Kauffmann, 1935, 1936). Perry *et al.* (1934) also suggested the preservation of such rejuvenated strains in mouse spleen dried *in vacuo* and stored in sealed ampoules. The increased content of the Vi antigen induced by these procedures does not, however, persist and on ordinary media the cultures soon revert to the less virulent form (Brown, 1936; Kauffmann, 1937). The great advantage derived from employing the strain Ty 2 is due to its outstanding ability to produce Vi antigen in maximum amount when maintained on plain agar.

The strain has constantly retained this property for a period of twenty years without a single passage through an animal or subculture on the more complicated media.

The strain Watson also retains its O inagglutinability in a remarkably constant manner. Cultures of this strain are decidedly less virulent than those of the strain Ty 2, and their content of the Vi antigen is correspondingly smaller. This can be shown either by quantitative absorption tests or by the precipitation technique suggested by Schütze (1936). The Vi titres established with the strain Watson in agglutination tests are about three times higher than those recorded for the strain Ty 2. On account of its inagglutinability by the O antibody and its sensitiveness in Vi agglutination the strain Watson serves as the test culture for the estimation of the Vi agglutination titre.

The strain "Ty 6S" (Felix & Petrie, 1938) is an antigenically "rough" variant, that is, devoid of O antigen, but otherwise resembles the smooth type. Suspensions of this culture are stable even in 5% saline and have been extensively employed in agglutination tests for nearly three years. Scholtens (1937), who used this strain in experimental work, suggested the name "Vi half-smooth form" for this type of antigenic variant of the typhoid bacillus. This variant is also very poor in H antigen and gives no agglutination with pure H serum, although the presence of small amounts of H antigen in the cultures is revealed when they are used for the immunization of rabbits or horses. A suspension of the strain Ty 6S represents indeed a pure reagent for the demonstration of the Vi antibody. It was hoped that by employing this culture the technique of the Vi agglutination reaction would be simplified in the same manner as that of O agglutination tests by the introduction of a pure reagent for the O agglutinin, namely, the permanent pure O variant "O 901" and later the alcohol-treated O suspension. However, the chief drawback in using the strain Ty 6S is its low sensitiveness in Vi agglutination. The Vi titres for the strain Ty 6S, like those for Ty 2, are about three times lower than those for the Watson strain. The culture Ty 6S cannot, therefore, be recommended for use in agglutination tests instead of the sensitive strain Watson, but it nevertheless serves as a very helpful control reagent when used in conjunction with the strain Watson.

Table II shows the agglutination reactions obtained with the various strains when used with pure H, O and Vi sera and also with the provisional standard serum. The degree of O inagglutinability of the strains Ty 2 and Watson is indicated by their reactions with the pure O serum, for it is seen that these strains react in the serum dilution 1 in 100 to the same degree as the strain O 901 reacts in the serum dilution 1 in 100,000, that is to say, the strains Ty 2 and Watson are about 1000 times less O agglutinable than the strain O 901. The relative Vi agglutinability is shown by their reactions with the pure Vi serum; the Vi agglutinability of the strain Watson is about three times that of the strains Ty 2 or Ty 6S. These results constitute the best criterion of the cultures being in proper condition in respect of their content of the Vi antigen.

Table II. *Showing the agglutination reactions obtained with the various strains of Bact. typhosum used in the titration of anti-typhoid sera.*

Readings: Black = H agglutination; Green = O agglutination; Red = Vi agglutination

Serum	Dilution	Strains				
		Ty 2	Watson	Ty 6S	O 901	H 901
Pure H serum	1 : 1000	+ ±	+ ±	-	-	+ + ±
Pure O serum	1 : 100	(±)	(±)	-		
	1 : 200	(±)	(±)	-		
	1 : 500	((±))	((±))	-		
	1 : 1000	((±))	((±))	-	+++	+++
	1 : 2000	-	-	-	+++	+++
	1 : 5000	-	-	-	+++	+++
	1 : 20,000	-	-	-	++	+ ±
	1 : 50,000	-	-	-	+	±
	1 : 100,000	-	-	-	(±)	-
	1 : 200,000	-	-	-	-	-
Pure Vi serum	1 : 200	+++	+++	+++	-	-
	1 : 300	++	+++	+++	-	-
	1 : 400	±	+++	+	-	-
	1 : 500	-	+++	(±)	-	-
	1 : 600	-	+++	-	-	-
	1 : 800	-	+++	-		
	1 : 1000	-	++	-		
	1 : 1200	-	+	-		
	1 : 1400	-	(±)	-		
	1 : 2000	-	-	-		
Saline controls 0.04 (% NaCl)	0.85	-	-	-	-	-
	2.5	-	-	-	-	-
	5.0	-	-	-	-	-
Provisional standard serum	1 : 100	+++	+++	+++		
	1 : 200	±	+++	+		
	1 : 300	-	+++	(±)		
	1 : 400	-	++	-		
	1 : 500	-	+ ±	-		
	1 : 600	-	+	-		
	1 : 800	-	(±)	+ ±		
	1 : 1000	-	-	+ ±		
	1 : 2000	-	-	+ ±		
	1 : 5000	-	-	+ ±		
	1 : 10,000	-	-	+ ±	+++	
	1 : 20,000		+ ±	+	++	
	1 : 30,000		+	±	±	
	1 : 40,000		+	+	(±)	
	1 : 50,000		±	±	((±))	
	1 : 60,000		±	±	-	
	1 : 100,000		(±)	(±)	-	
	1 : 200,000		-	-	-	

Reading after 24 hr. (2 hr. incubation at 37° C. and thereafter at room temperature).

+ + + =strongest degree of agglutination; supernatant fluid completely clear.

+ + ± to + =degrees of incomplete agglutination; supernatant fluid turbid.

± =weakest degree of agglutination which could be estimated with the naked eye.

(±) =trace } estimated by means of magnifying lens.

((±)) =faint trace }

The titre of Vi agglutination =the highest dilution which shows a reaction corresponding to + (naked-eye reading).

The titre of O agglutination =the highest dilution which shows a reaction corresponding to ((±)) (reading by means of magnifying lens).

Control tests of this kind must give results similar to those recorded in Table II if the test cultures are to be used successfully for the purposes specified in Table I.

It is obvious from Table II that so long as the culture of the strain Watson retains its O agglutinability at the level stated above, namely, about 1000 times lower than that of the strain O 901, any granular small-flaked agglutination above this titre obtained with this strain will indicate the presence of Vi antibody. If the further precaution is taken to assign as the Vi titre of a serum the dilution which gives an agglutination reaction of a degree corresponding to the symbol +, as estimated with the naked eye, so that weaker degrees are disregarded although they may be read and recorded in the protocols, it will be found after some experience that it is possible to make a correct estimation of the Vi agglutinin titre of a serum which also contains O antibody of high titre.

The floccular H agglutination, which is present up to very high dilutions of the standard serum and also of nearly every sample of therapeutic anti-typhoid serum, does not represent any real source of error in the correct reading of the granular Vi agglutination. In this connexion the following precaution should be taken. The cultures of the strain Watson should be grown in such a manner that about half of the organisms are inagglutinable by H antibody (see p. 761). This is readily achieved by colony selection, since there seems to be an antagonism between the maximum formation of Vi antigen and the full development of flagella (Kauffmann, 1935; Giovanardi, 1938). It is seen from Table II that the H agglutination reactions of the strains Ty 2 and Watson are less complete than that of the Vi-free strain H 901. This property is so constant that a marked increase in the H agglutinability of the suspension may serve as a useful warning of the occurrence of some variation in the Vi content of the cultures. Detre (1937) has suggested the employment of a "defloccularized" suspension of the strain Watson with a view to eliminating H agglutination.

It is obvious that the best reagent for Vi agglutination tests would be a variant of the type of the strain Ty 6S provided that it were endowed with a degree of Vi agglutinability equal to or greater than that of the strain Watson. According to Scholtens (1937) variants of this type can be produced at will by the action of a suitable bacteriophage. While this paper was being prepared, a culture of a naturally occurring variant of this type was received from Major S. S. Bhatnagar, I.M.S. The properties of this variant, which has been designated "Vi I", are described in a recently published paper (Bhatnagar *et al.* 1938). Although cultures of this strain contain a very small quantity of the H and the O antigen, detectable by the immunization of rabbits, the suspensions seem to be agglutinable solely by the Vi antibody. Preliminary tests with this culture over a period of a few weeks indicate that Bhatnagar's strain "Vi I" may prove to be a very useful reagent for Vi agglutination.¹

¹ Cultures of Bhatnagar's strain "Vi I" can be obtained from the present writer or from the National Collection of Type Cultures, Lister Institute, London, S.W. 1, England.

THE PRESERVATION AND MAINTENANCE OF THE CULTURES USED IN THE TESTS

The simplest method of preserving the cultures is to employ sealed or waxed agar-stab cultures; these can be stored in the dark at room-temperature for a long time, even for many years. It is, however, essential to use for this purpose agar medium made with broth from meat extract, instead of from fresh meat, in order to avoid the presence in the medium of fermentable substances. Agar medium prepared in the following way has given satisfactory results over a period of many years:

- 1000 c.c. tap water.
- 20 g. agar powder.
- 10 g. Bacto peptone.
- 10 g. Lab Lemco (meat extract).

Steam for 30 min.; add 12 c.c. *N*/1 sodium hydrate. Cool to 60° C. Add two whites of egg. Steam until clear. Filter through lint. Adjust reaction to pH 7.4. Tube and autoclave 30 min. at 120° C.

When a batch of agar-stab tubes is prepared and stored for some time before being inoculated, it is desirable to re-autoclave the required number of tubes a day or two before they are used, and to remove the water of condensation completely by keeping the tubes in the incubator or hot room.

It is especially emphasized that the peptone-Lemco agar should be used *exclusively* for keeping cultures in waxed stabs, whereas the agar used for surface growth should be made from fresh meat broth and should not contain any meat extract or commercial peptone.

The cultures employed in the various tests are grown on a trypsin-digest agar of a pH of 7.4; they are transplanted daily, including also on Sunday, and are kept in the incubator at 37° C. The constancy of the temperature at which the cultures are grown must be carefully watched and the time during which the cultures are manipulated at room temperature must be reduced to a minimum (Felix *et al.* 1934).

The cultures of all the strains used are plated out on trypsin-digest agar medium at least once a week and not less than ten or twelve carefully selected colonies, all showing the characters of perfect "smoothness", are seeded together on an agar slope. The strains Ty 2, Watson and H 901 are grown on slopes which contain water of condensation, while the strains O 901 and Ty 6S are kept on agar slants which are free from water of condensation. When selecting smooth colonies of the Vi strains it has been found useful to pay attention to the opacity of the colonies as observed by transmitted light: opaque colonies are composed of bacteria with a high content of the Vi antigen, whereas translucent colonies are formed by bacteria that are deficient in or even devoid of the Vi antigen. Similar observations have been recently published by Giovanardi (1938).

When cultures of one of the Vi strains, in spite of repeated colony selection,

fail to react with the control sera in the manner illustrated in Table II, they are discarded and replaced by a culture derived from one of the agar stabs that are being kept in stock. A considerable number of stab cultures of each of the three Vi strains used in the tests should, therefore, be prepared at the time when the cultures are known to be in the proper condition in respect of their content of the Vi antigen.

THE TECHNIQUE OF THE AGGLUTINATION TESTS

Few technical details need be described here. Any kind of technique of the agglutination reaction is capable of giving trustworthy results, since the titration of both the Vi and the O antibody is based on comparison of the reactions produced by the sera under test with those produced by the provisional standard serum.

Most of the results discussed in this paper and in previous work have been derived from agglutination tests performed with fresh saline suspensions of living bacteria taken from 24 hr. agar-slope cultures (see Felix & Pitt, 1934*a*). The details recorded in Table II also refer to such tests. Within recent months killed suspensions, preserved with 0.2% formalin, have been employed in parallel with suspensions of living organisms, and the results obtained by the two reagents have been for all practical purposes almost identical.

With regard to the O agglutination test the reader is referred to the recent paper by Felix & Gardner (1937) which contains a number of recommendations for the standardization of this test. It may be added here that the O agglutinin titres established with preserved suspensions after incubation at 50–52° C. for 20–24 hr., usually correspond closely to those established with the living bacteria after 2 hr. incubation and a reading after a further 20 or 22 hr. at room temperature.

It is seen from Table II that the "titre" of O agglutination has been assigned as the highest dilution of the serum which shows a "faint trace" reaction, corresponding to the symbol ((+)), which is estimated by means of a magnifying lens. In earlier work on the diagnostic use of O agglutination in cases of enteric fever the strongest degree of agglutination, corresponding to the symbol + + +, had been employed to indicate the "titre". The dilution which gives a ((+)) reaction is usually ten times higher than that which gives a + + + reaction.

The end-point now suggested has been adopted for the following reasons:

(a) Since Gardner (1929) first recommended for the "O titre" the reading of "the last trace of clumping visible with a watchmaker's lens", the procedure has gradually come into almost general use.

(b) The "trace" reading is probably more easy to define than that of any other degree of partial agglutination. It is, therefore, to be expected that greater uniformity in readings by various observers can be secured by means of this definition of the "titre".

(c) The very weak agglutination reactions which the Vi strains give with a pure O serum can best be compared with the reactions which the strain O 901 gives when the "trace" readings for the various suspensions are considered.

With regard to the Vi agglutination test the following points must be specifically mentioned:

(1) The temperature of incubation should be 37° C., whether living bacteria or preserved suspensions are employed. The tubes should be incubated for 2 hr. at 37° C. and the final reading taken after a further 20 or 22 hr. at room temperature.

(2) Suspensions of the strain Watson prepared from 24 hr. agar cultures and preserved with 0.2% formalin maintain their inagglutinability by O antibody and their sensitiveness to Vi antibody for about six months.

(3) Similarly prepared suspensions of the "half-smooth" strain Ty 6S tend to deteriorate rather sooner, that is, after storage for about four months.

The reader is referred to the paper by Felix & Gardner (1937) for details of the preparation of the formolized suspensions.

(4) The Vi agglutinin titres against the formolized suspensions of the strains Watson and Ty 6S are equal to or slightly higher than those against the corresponding fresh suspension of the living bacteria.

(5) Suspensions of the strain Watson, whether preserved or fresh, should be standardized to give a bacterial content of between 400 and 600 million organisms per c.c. of the final agglutinating mixture. This corresponds to an opacity about 50% greater than that recommended for agglutination tests in general (Felix & Gardner, 1937) and is necessitated by the fact that about half the number of organisms contained in the agglutinating mixtures is flocculated by the H antibody which is usually present in the serum (see p. 758). Thus the supernatant fluid resulting from H agglutination alone is still sufficiently turbid to allow a naked-eye estimation of the degree of clearance due to Vi agglutination.

(6) It is known that the flagellar H antigen, when present in formolized suspensions, inhibits the reaction between the O antigen and the O antibody (Felix & Olitzki, 1928). The most probable explanation of the phenomenon seemed to be the one suggested by Craigie (1931), according to which the hardened flagella mechanically separate the bodies of the bacteria, and thus inhibit O agglutination. It is of theoretical and practical interest to note that the Vi agglutination of formolized suspensions is not inhibited by the presence of flagella. On the contrary, the velocity of the Vi agglutination is greater with the formolized suspension than with the fresh suspension of living organisms and the end-titre of the reaction, too, is rather higher with the preserved suspension. Since both the Vi and the O antigen are "somatic" antigens, the different effects of formolized flagella observed in the two types of agglutination reaction seem to call for some explanation other than that suggested by Craigie (1931).

THE ESTIMATION OF THE Vi ANTIBODY

(a) The agglutination test

Suitable dilutions of the sera to be examined are tested against suspensions of the strains Watson and Ty 6S; the provisional standard serum and control sera similar to those described in Table II are tested at the same time.

The Vi titre of a serum is the dilution of the serum which gives Vi agglutination against the strain Watson to a degree equal to that produced by the dilution 1 in 600 of the standard serum.

The degree of accuracy of the estimation depends upon the experience of the investigator. In numerous tests on the provisional standard serum carried out at this laboratory during the past three years, the deviation in the readings of its Vi titre has not exceeded 20% above or below the reading shown in Table II.

*(b) The mouse-protection test with living typhoid bacilli**(1) Preliminary test of virulence of the strain Ty 2: the determination of the test dose of the culture.*

Cultures of the strain Ty 2, grown on trypsin-digest agar slopes at 37° C. for not more than 16 hr. are employed. The growth is suspended in and further diluted with Ringer solution of a pH of from 7·6 to 7·4, the temperature of which has been brought to 37° C.; the bacterial content is estimated by opacity. The number of organisms required for the various doses is always contained in 0·5 c.c. and this quantity is injected intraperitoneally, with the least possible delay, into male mice weighing 16–20 g. The same syringe is used for the injection of the various dilutions of bacterial suspension, commencing with the one which contains the smallest dose, but each of the dilutions is prepared in a quantity in excess of that needed, so that the syringe can be repeatedly rinsed with each of the dilutions. The mice are shaved on the abdomen and occasional accidents, such as loss of a fraction of the volume injected or injury to the animal, are recorded.

Mice which succumb to the infection die, with very few exceptions, within 48 hr., and the majority within 24 hr. following the intraperitoneal injection. The survivors, nevertheless, are kept under observation for five days.

Table III. *The determination of the test dose of the culture Ty 2*

Group	Number of organisms in dose	Number of mice in group	Number of mice which died
1	80×10^6	10	10
2	70×10^6	10	9
3	60×10^6	10	7
4	40×10^6	10	5

Table III shows that the dose of 80×10^6 organisms represented 1 M.L.D. of the culture employed in the test which is recorded in the table. This is the

degree of virulence of the strain Ty 2 usually established with a culture which in agglutination tests with pure O and pure Vi sera gives reactions similar to those described in Table II. From a perusal of the tables published in previous papers (Felix & Pitt, 1934*b*, 1935; Felix & Bhatnagar, 1935) it will be seen that the M.L.D. of the test cultures of this strain was almost invariably a dose of 80×10^6 organisms. Occasionally the Vi content of the culture may be so high that 1 M.L.D. is contained in a smaller dose, sometimes even in a dose of 50×10^6 organisms; this, however, is quite exceptional.

The preliminary test of the virulence of the culture should be carried out two or three days prior to the employment of the culture for the preparation of the test dose in the comparative protection tests. The test dose should be about 3 M.L.D. and should be calculated according to the results observed in the preliminary test 48 hr. after the intraperitoneal injection.

From the experience of the test acquired during the past few years it would appear that uniform results of the comparative mouse-protection tests are obtained so long as 1 M.L.D. of the test culture is represented by a dose not greater than 100×10^6 organisms. If the culture falls below this level of virulence it should be discarded and replaced by a fresh agar-stab culture.

(2) *Comparative protection tests to determine the potency ratio of the serum under test and the provisional standard serum.*

The following table shows the results of two experiments carried out with a sample of therapeutic anti-typhoid serum (no. 23) and the reconstructed provisional standard serum.

The mice are given the serum intramuscularly in the thigh and the test dose intraperitoneally 48 hr. later. The two sites are prepared for the injections by shaving both of them at the same time. A titration of the test dose in groups of control mice is included in each experiment.

The potency of the provisional standard serum in the mouse-protection test was known from previous experience; the requisite dose of the concentrated serum no. 23, was calculated from its Vi agglutinin titre, which was known to be five times that of the provisional standard serum. The two experiments were carried out at different times and the test doses were prepared from cultures of the strain Ty 2 that differed in virulence to the extent shown in the table. In the first experiment the dose of 80×10^6 organisms represented 1 M.L.D. whereas in the second experiment this proved to be the dose of 70×10^6 organisms.

It is seen from Table IV that the percentage of serum-treated mice that survived was much lower with the test dose of 4 M.L.D. than with the test dose of 3 M.L.D. However, the amount of protection afforded by a dose of 0.04 c.c. of the concentrated serum no. 23 was in both experiments practically the same as that afforded by a dose of 0.2 c.c. of the provisional standard serum. The doses of the two sera that are approximately equivalent in protective action are in the inverse ratio of the Vi agglutinin titres of the sera.

Table IV. *Comparative protection tests*

Exp. 1. Test dose: 3 M.L.D. from strain Ty 2.

Serum	Agglutinin titre of anti-bodies present in the serum		Dose of serum in c.c.	Number of mice tested by intra-peritoneal injection of 240×10^6 organisms		Percentage of survivors
	Vi	O		Tested	Survived	
Provisional standard serum	1 : 600	1 : 50,000	0.2	20	14	70
No. 23 concentrated	1 : 3000	1 : 150,000	0.04	20	15	75
Controls with normal mice:						
Group receiving	100 $\times$ 10 ⁶ organisms			10	0	
" "	80 $\times$ 10 ⁶ organisms			10	0	
" "	70 $\times$ 10 ⁶ organisms			10	2	
" "	60 $\times$ 10 ⁶ organisms			10	4	
" "	40 $\times$ 10 ⁶ organisms			10	6	

Exp. 2. Test dose: 4 M.L.D. from strain Ty 2.

Serum	Agglutinin titre of anti-bodies present in the serum		Dose of serum in c.c.	Number of mice tested by intra-peritoneal injection of 280×10^6 organisms		Percentage of survivors
	Vi	O		Tested	Survived	
Provisional standard serum	1 : 600	1 : 50,000	0.2	20	9	45
No. 23 concentrated	1 : 3000	1 : 150,000	0.04	20	8	40
Controls with normal mice:						
Group receiving	100 $\times$ 10 ⁶ organisms			10	0	
" "	80 $\times$ 10 ⁶ organisms			10	0	
" "	70 $\times$ 10 ⁶ organisms			10	0	
" "	60 $\times$ 10 ⁶ organisms			10	2	
" "	40 $\times$ 10 ⁶ organisms			10	4	

(3) *The effect on the survival rate of varying the dose of serum administered in the mouse-protection test.*

The results of the experiment recorded in Table V show that a difference in the dose of the provisional standard serum of the order of 25 % is indicated by a difference in the survival rates when groups of 20 mice are employed for each dose.

Table V also shows the extent to which the results of the mouse-protection test with the virulent culture are independent of the presence in the serum of O antibody of high titre. Two sera were included in the test which contained O antibody of equal titre; the serum no. 6, which had been specially prepared to serve as a pure O serum for clinical trials, was devoid of the Vi antibody, whereas the serum no. 23 had a Vi agglutinin titre of 1 in 3000, in addition to its O titre of 1 in 150,000. The serum no. 6 was given in the dose of 0.2 and 0.4 c.c. to groups of 10 mice, that is to say, the amounts of O antibody given to these mice were, respectively, five times and ten times the amount contained

Table V. *The degree of accuracy of the mouse-protection test*

Serum	Agglutinin titre of anti- bodies present in the serum		Dose of serum in c.c.	Number of mice tested by intra- peritoneal injection of 240×10^6 organisms		Percentage of survivors
	Vi	O		Tested	Survived	
Provisional standard serum	1 : 600	1 : 50,000	0.2	20	15	75
			0.15	20	12	60
No. 23 concentrated	1 : 3000	1 : 150,000	0.04	20	14	70
No. 6 concentrated	1 : 20	1 : 150,000	0.2	10	0	0
			0.4	10	1	10
Controls with normal mice:						
Group receiving 100×10^6 organisms				10	0	
„	„	80×10^6 organisms		10	0	
„	„	70×10^6 organisms		10	1	
„	„	60×10^6 organisms		10	3	
„	„	40×10^6 organisms		10	5	

in the dose of 0.04 c.c. of the serum no. 23. It is seen from Table V that under the conditions of the test the effect of the pure O serum of high titre is negligible.

It should be again emphasized that the virulence of the test culture, as defined in this section, is the essential pre-requisite of the method of titrating the Vi antibody in the mouse. It has been shown by Topley *et al.* (1937) that the O antibody confers a high degree of protection against infection with cultures that are less virulent and less O resistant than the strain Ty 2. The minimal (average) lethal dose of the "rejuvenated" Rawlings strain employed in the experiments of Topley *et al.* (1937) is stated by these workers to have been "rather less than 100 million bacilli"; the corresponding figure for the strain Ty 2 is about 40 million bacilli. The test dose of 500 million organisms of the rejuvenated Rawlings strain, which Topley *et al.* (1937) used throughout, killed as a rule only 90% of the normal control mice; when the strain Ty 2 is employed, there is hardly ever a survivor amongst the control mice inoculated with either the full test dose or half the test dose, and very rarely is there a survivor in the group which has received a third of the test dose.

The question whether the administration of the serum by the intravenous or the subcutaneous route materially alters the conditions of the test described here has not been investigated. It would appear from the papers recently published by Henderson & Morgan (1938) and Boivin & Mesrobianu (1938*b*) that the method of injecting the test culture together with mucin is entirely unsuitable for the titration of the Vi antibody, since this treatment apparently deprives the bacilli of their O resistance.

(4) *The provisional unit of the Vi antibody.*

A provisional unit of the Vi antibody has been adopted as the basis for calculating the proportional amounts of anti-typhoid serum obtained from different horses that can be pooled for the preparation of the concentrated

final product. The amount of Vi antibody present in 0.2 c.c. of the reconstructed provisional standard serum has been taken as the provisional unit of the Vi antibody. It has been shown in Tables IV and V that, under the conditions of the mouse-protection test here described, the dose of 0.2 c.c. of the serum usually protects about 50–75% of mice weighing 16–20 g. against a test dose of 3 M.L.D. of the virulent strain Ty 2.

THE ESTIMATION OF THE O ANTIBODY

(a) *The agglutination test*

The O titre of a serum under test is the dilution of the serum which gives agglutination against the strain O 901 of a degree equal to that produced by the dilution 1 in 50,000 of the provisional standard serum.

In numerous tests on the provisional standard serum carried out during the past three years the highest readings of the O titre were recorded as the dilution 1 in 60,000, and the lowest 1 in 40,000; the mean value of 1 in 50,000 was recorded in most instances. These titres were determined against fresh saline suspensions of the living culture of the strain O 901. It is probable that the deviation in the readings will not be greater when preserved O suspensions are employed.

(b) *The mouse test for the neutralization of the toxic action of killed typhoid bacilli*

(1) *Preliminary test of the toxic action of doses of killed typhoid bacilli: the determination of the test dose of "endotoxin".*

While the significance of the protection test with living bacilli wholly depends upon the employment of a specially selected test culture, the test dose of killed bacilli used in the neutralization test may be prepared from any smooth strain of *Bact. typhosum* that contains its full quota of the O antigen. It has been shown in a previous paper (Felix & Pitt, 1934*a*) that heat-killed suspensions of virulent strains are not more toxic for mice than those of strains of lowest virulence. This observation has been amplified by numerous experiments with strains which are representative of all the types of antigenic variants of *Bact. typhosum* that have been described hitherto. It is not possible to discuss here the details of these experiments, but they may be summarized as follows:

(i) The toxicity for mice of suspensions of typhoid bacilli killed by heating at 58° C. is independent of the presence in the bacilli of either the H or the Vi antigen; the toxicity is determined by the quantity of the O antigen contained in the bacilli.

(ii) Alcohol-treated suspensions of bacilli which possess both the O and the Vi antigen are not more toxic than those containing only the O antigen. This is of particular interest since it is known that the treatment with alcohol does

not destroy the agglutinogenic and immunogenic properties of the Vi antigen (Felix & Pitt, 1936; Felix & Petrie, 1938).

(iii) The toxicity of the suspensions is practically the same after heating for 2 hr. at 58° C. or for half an hour at 100° C.

These findings would appear to be in general agreement with the observations on the properties of various antigenic fractions of *Bact. typhosum* that have been prepared by different methods by Topley *et al.* (1937), Henderson & Morgan (1938) and Boivin & Mesrobian (1938a). It is probable that a dried purified antigenic fraction prepared from a variant of the type of the strain O 901 may ultimately be found to be the most suitable reagent for the preparation of the test dose of "endotoxin".

In the experiments recorded in the following tables the test suspensions used were prepared throughout in the following manner: the culture of the strain O 901, which has been carefully examined for "smoothness", is grown for 24 hr. at 37° C. on a number of Roux bottles containing trypsin-digest agar and the growth is washed off and diluted with saline to give a suspension which contains 40,000 million organisms per c.c. The suspension is sterilized by heating for 2 hr. at 58° C. and is then stored in the cold room. The sterility tests are discarded after five days' incubation; nevertheless the suspension can be used after 24 hr. for the preliminary test of the toxic action, provided the sterility tests at that time are negative. Suitable dilutions of the suspension are prepared with saline, so that the doses injected are always contained in 0.5 c.c. and this quantity is injected intraperitoneally into male mice weighing not more than 14–16 g.

Table VI. *The determination of the test dose of killed bacilli*

Vaccine from strain O 901 heated for 2 hr. at 58° C.

Group	Number of organisms in dose	Number of mice in group	Number of mice which died
1	20,000 × 10 ⁶	10	10
2	16,000 × 10 ⁶	10	9
3	12,000 × 10 ⁶	10	8
4	8,000 × 10 ⁶	10	5

The clinical picture observed in the mice that receive the large doses of killed bacilli differs altogether from that which follows the injection of the comparatively small doses of living virulent bacilli. In the latter case the mice do not show any symptoms of illness during the first few hours after the injection, although the majority of the animals that succumb to the infection die within 24 hr. In contrast to this, practically every mouse that is given the dose of killed bacilli shows definite symptoms of illness within the first few hours after the injection, irrespective of whether the animal ultimately succumbs or survives. Deaths resulting from doses of killed bacilli may occur as late as three or four days after the injection.

The M.L.D. of the test suspension for mice weighing 14–16 g. will usually be found to be a dose of 20,000 million organisms (see Table VI), although oc-

casionaly a smaller dose may be found to kill every mouse in the group, when groups of 10 mice are employed for each dilution of the suspension.

It is not practicable to administer much more than 20,000 million killed typhoid bacilli to a mouse; accordingly, in attempting the determination of the O antibody by neutralization tests in mice it has only been possible to test the effect of doses of serum against one lethal dose of killed organisms. The defects inherent in such a procedure are well known and it is hoped that a dried purified antigenic fraction from a suitable strain will provide a more satisfactory reagent for the preparation of the test dose.

(2) *Comparative neutralization tests to determine the potency ratio of the serum under test and the provisional standard serum.*

The mice are prepared for the injections by shaving the thigh and abdomen; the doses of serum are injected intramuscularly and the test dose is given intraperitoneally 48 hr. later.

Table VII. *Comparative neutralization tests*

Test dose: 1 M.L.D. of vaccine from strain O 901 heated for 2 hr. at 58° C.

Serum	Agglutinin titre of antibodies present in the serum		Dose of serum in c.c.	Number of mice tested by intraperitoneal injection of $20,000 \times 10^6$ organisms		Percentage of survivors
	Vi	O		Tested	Survived	
Provisional standard serum	1 : 600	1 : 50,000	0.4	10	6	60
No. 19 concentrated	1 : 3000	1 : 100,000	0.2	10	5	50
No. 18 concentrated	1 : 1800	1 : 150,000	0.14	10	7	70
Controls with normal mice:						
Group receiving	$20,000 \times 10^6$ organisms			10	0	
" "	$16,000 \times 10^6$ organisms			10	1	
" "	$12,000 \times 10^6$ organisms			10	2	

In the experiment recorded in Table VII the reconstructed provisional standard serum was given in the dose of 0.4 c.c., which from previous experience was known to protect about 50–75% of the mice against 1 M.L.D. of the test suspension. The doses of the concentrated sera nos. 19 and 18 were adjusted to one-half and one-third of the dose of the provisional standard serum, according to the O agglutinin titres of the two sera.

For the reasons stated in a subsequent section the neutralization tests in the mouse have been applied much less in routine work than the mouse-protection tests, and groups of 10 mice only were used in the neutralization tests. Taking into account the small number of animals, the survival rates in the three groups of serum-treated mice shown in Table VII may be regarded as being in fairly good agreement. It is seen from the table that the doses of the three sera that are approximately equivalent in neutralizing action are in the inverse ratio of the O agglutinin titres of the sera.

A noteworthy feature, which is often observed in the mice protected with an adequate quantity of O antibody, is the complete absence of those early symptoms of illness which have been referred to previously. The period of observation of the survivors in the neutralization test is five days from the injection of the test suspension.

(3) *The effect on the survival rate of varying the dose of serum administered in the neutralization test.*

Table VIII shows that a difference in the dose of the provisional standard serum of the order of 25% is reflected by a difference in the survival rate, even when groups of only 10 mice are employed for each dose of serum. The accuracy of the method is, of course, increased by the use of larger groups of mice.

Table VIII. *The degree of accuracy of the neutralization test in the mouse*

Test dose: 1 M.L.D. of vaccine from strain O 901 heated for 2 hr. at 58° C.

Serum	Agglutinin titre of anti-bodies present in the serum		Dose of serum in c.c.	Number of mice tested by intraperitoneal injection of $20,000 \times 10^6$ organisms		Percentage of survivors
	Vi	O		Tested	Survived	
Provisional standard serum	1 : 600	1 : 50,000	0.4	10	7	70
			0.3	10	5	50
No. 23 concentrated	1 : 3000	1 : 150,000	0.15	10	6	60
No. 6 concentrated	1 : 20	1 : 150,000	0.15	10	7	70
Controls with normal mice:						
Group receiving $20,000 \times 10^6$ organisms				10	0	
" " $16,000 \times 10^6$ organisms				10	2	
" " $12,000 \times 10^6$ organisms				10	3	

For the sake of completeness the two sera nos. 23 and 6, which had been employed in the protection test described in Table V, were also included in the neutralization test recorded in Table VIII. It is evident from the table that the value of a serum in the neutralization test is determined by its content of the O antibody and is independent of its content of the Vi antibody.

(4) *The provisional unit of the O antibody.*

A provisional unit of the O antibody has been adopted for routine purposes in connexion with the process of preparation of the concentrated therapeutic serum. The quantity of O antibody present in 0.4 c.c. of the reconstructed provisional standard serum has been taken as the provisional unit of the O antibody. This dose of the serum usually protects about 50–75% of mice weighing 14–16 g. against 1 M.L.D. of the test suspension prepared from the strain O 901.

THE PRACTICAL APPLICATION OF THE VARIOUS TESTS

(a) The routine titration of samples of serum taken in the course of preparation of therapeutic anti-typhoid sera

The results of the experiments described in this paper seem to warrant the conclusion that the agglutinin titres of the Vi and the O antibody provide an accurate and trustworthy measure of the biological activities of the two antibodies. So far as the Vi antibody is concerned this statement must be qualified by the proviso that the Vi antibody must not belong to the variety characterized by the peculiar "functional deficiency" which has been described by Felix & Bhatnagar (1935). Provided it is known that the Vi antibody has been elaborated in response to immunization with the "natural" Vi antigen, as it is contained in the living bacterial cell, the Vi agglutinin titre as well as the O agglutinin titre can be accepted as the final measure of the therapeutic activities of the two antibodies.

The immunizing procedures now employed in the preparation of therapeutic anti-typhoid serum at the Serum Department of the Lister Institute have been described in a previous paper (Felix & Petrie, 1938). The routine titration of samples of the natural serum from each separate bleeding consists of agglutination tests alone, the results of which are available 24 hr. after the samples have been taken. The concentrated final product, too, is usually subjected to agglutination tests alone. Occasionally the precaution is taken to titrate a sample of the final globulin solution in the mouse-protection test for the estimation of the Vi antibody.

(b) The official control of the potency of therapeutic anti-typhoid sera

The position is quite different when the potency of therapeutic sera from various sources is estimated. In view of the possibility of some of the sera containing Vi antibody of the "deficient" variety, the mouse-protection test for the estimation of the Vi antibody is clearly indispensable. The Vi agglutination test will supply a useful orientating result, which will serve as the basis for calculating the doses of serum to be employed in the mouse-protection test.

It has not yet been shown that the O antigen is also liable to an alteration similar to that of the Vi antigen which is responsible for the formation of an antibody characterized by "functional deficiency". This point has been investigated by examining sera from horses which had been immunized with the following kinds of vaccine or bacterial fractions containing the O antigen: suspensions of living virulent bacilli; suspensions of bacilli killed by heating at 58° C. or by treatment with alcohol; the antigenic fractions prepared according to the methods of Raistrick & Topley (1934) and of Morgan (1937). The potency of these sera as determined by the neutralization test in the mouse

ran, on the whole, parallel with the titre of O agglutination, thus indicating that the O antibody contained in each of the sera was endowed with full functional efficacy.

It is, however, possible that some other form of treatment of the O antigen may lead to the elaboration by the immunized animal of an O antibody of "functional deficiency". It is also conceivable that some method of concentrating the serum other than that used at the Serum Department of the Lister Institute (see Felix & Petrie, 1938) may cause an alteration in the ratio of the neutralizing value to the O agglutinin titre. For these reasons it appears to be necessary for purposes of official control to carry out the neutralization test in the mouse. Again, the agglutination test will serve as a guide to the selection of the doses of serum to be used in the mouse test.

THE PERMISSIBLE MINIMAL POTENCY OF THERAPEUTIC ANTI-TYPHOID SERA

It has been possible to maintain the potency of the batches of therapeutic anti-typhoid serum prepared during the past year at an almost constant level. The minimum potency of the serum has been stated in a previous paper (Felix & Petrie, 1938) in terms of "titres" derived from the Vi and the O agglutination test, the mouse-protection test and the neutralization test. In terms of the two provisional units, as defined in the present communication, the minimum potency of the therapeutic serum is as follows:

1 c.c. of the serum contains 25-30 provisional units of the Vi antibody.

1 c.c. of the serum contains 6-7.5 provisional units of the O antibody.

For comparison the agglutination titres are again stated here:

Vi titre: 1 : 3000 to 1 : 3600 against the strain Watson.

O titre: 1 : 120,000 to 1 : 150,000 against the strain O 901.

The average dosage recommended for the treatment of adults in cases of typhoid fever of moderate severity is 99 c.c. ($= 3 \times 33$ c.c.) of the concentrated anti-typhoid serum as issued at the present time. This dose contains approximately: 2500-3000 provisional units of the Vi antibody, and 600-750 provisional units of the O antibody. From the results of clinical trials so far available it would appear that this dose represents the permissible minimum.

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