ANTITYPHOID SERA.¹

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INTRODUCTORY.

It is of great interest and importance to determine, if possible, whether an antityphoid serum of real and marked therapeutic value can be prepared from the horse, or other large animal, and to define the conditions of such preparation.

Professor Ehrlich has concluded that the difference in species between the animal immunised and the animal to be protected or cured forms a barrier to success of the gravest importance in the production of antityphoid, antistreptococcic, antipneumococcic, and other antimicrobial sera. And certain evidence has, no doubt, been adduced in support of such a view. But the striking and brilliant theories of Ehrlich are not as yet sufficiently established. And it appears somewhat premature, at any rate, to attribute our want of success entirely to this difference in species.

Certainly these differences do not prevent a general valency of antitoxic sera. And until very strong evidence to the contrary is submitted we may reasonably continue to suppose that, of a series of species so closely similar to one another in their physiology as are the mammalia, those susceptible to the same micro-organisms, reacting to them by largely similar symptoms and capable of active immunisation against them, produce, in the process of immunisation, "protective bodies," differing by so little, if at all, as to be largely interchangeable, and therefore capable of therapeutic use. For though all recent researches show the necessity of recognising clearly a distinction between the principles governing the serum therapy of typhoid fever and that of diphtheria, or tetanus for example,—since we have to deal in the former with microbial invasion, but in the latter chiefly with the absorption of toxic products,—yet, as between species in their relation to typhoid, we may probably consider that the differences between the sera of immunised individuals are those of degree rather than those of kind; that it is, in fact, the quantity of protective material, or in other words the strength per cubic centimetre of the serum, that requires to be increased, and not an entirely new element

¹ Abbreviated from an M.D. Thesis, written and submitted April 1900.

or character to be introduced. And certainly good results have actually already been obtained in man, in spite of differences of species.

We are aware, of course, that different animals vary in their susceptibility to any given organism. And it is evident that the serum of a less susceptible animal, artificially immunised against that organism, will be less protective than that of a more susceptible animal brought to the same degree of immunisation; since the pre-existing basis of natural immunity, which is not transferable by transference of serum, is higher in the former than in the latter, and therefore the artificial transferable immunity lower in the first case than in the second. That is to say, for the same degree of immunity induced, a more strongly protective serum can be obtained from the more susceptible species than from those less so; and therefore, for man, more easily from those animals most nearly allied to him in constitution and susceptibilities. But it does not follow that sera of suitable valency and therapeutic use for man can only be obtained from his nearer allies in the animal series, nor that these should be employed in preference to others, if the practical difficulties are thereby unreasonably increased. It will be admitted, moreover, that in typhoid fever, for example, there is a toxic element of very considerable importance. It follows, therefore, that even if the position of Professor Ehrlich were admitted, it would still remain a possibility to obtain from animals a serum which would antagonise the toxic elements of the disease, though it might not directly affect the bacteria themselves.

IMMUNISATION AGAINST TYPHOID INFECTION.

The experiments of Sanarelli, Beumer and Peiper, Remlinger and Schneider, and others have shown that animals react to suitable typhoid infection in a manner closely similar to man. And many observers have found that the sera of immunised animals or of convalescent man are protective for other animals, though they differ considerably among themselves as to whether their properties are antitoxic, or antimicrobial, or both (Brieger, Kitasato and Wassermann, Chantemesse and Widal, Pfeiffer and Kolle, Bruschetti and Stern, Funck). Bokenham, moreover, prepared an antityphoid serum of considerable potency from the horse in 1897. His method of immunisation followed the plan of Cartwright Wood, for producing a rapid formation of antitoxine and a high degree of resistance to the toxine of diphtheria. By filtration of cultures he obtained a fluid innocuous to guinea-pigs, giving no symptoms after injection, except some rise of temperature. This fluid contained a substance chemically similar to one found by himself and Fenwick in the spleens of typhoid patients in a previous research.

Two horses were treated. The first received injections of the non-toxic filtrate, subsequently injections of killed cultures, and later alternating doses of the two. The second was injected with killed cultures only. The first horse remained well and was but little affected by the injections, the second reacted violently to each dose. The serum of the former gave a rapid agglutinative reaction, that of the latter was far less active. The susceptibility to living cultures of the first horse was far less than that of the second.

The non-toxic filtrate was therefore capable of causing protection, and the serum obtained had a "neutralising" power for typhoid bacilli when the two

were mixed and injected together into a guinea-pig. It had also a protective power, if injected forty-eight hours or more before the typhoid bacilli, though larger doses were required. It had also a curative action, if injected in large doses two or three hours after the infecting injection. It gave no protection against toxic filtrates of dead cultures. Experiments in the same direction have been carried on for some years by Professor Tavel in Bern, and a serum had been prepared and tested clinically with encouraging results.

The work which I undertook at Professor Tavel's suggestion was to determine as nearly as possible how far the value of the serum produced was affected by the variety of *Bacillus typhosus* employed, and what modifications, if any, resulted from the employment of several varieties, either together or in succession.

The idea of obtaining a "polyvalent" serum had been suggested by Bokenham in 1898, in connection with the preparation of anti-streptococcic sera. He pointed out that while it is admitted that a given culture of streptococcus is able, under varying conditions, to give rise either to erysipelas, or to pus formation, or to a general septicæmia or pyæmia, yet we cannot assume that all streptococci possess this ability to modify their pathogenetic action. And he adduced the example of the streptococcus associated with the condition known as "strangles" in the horse. This variety of streptococcus is specific for the horse, but pathogenetic for no other species of animal, so far as is known. From these facts he concluded that, even if an antistreptococcic serum be prepared of protective and curative value for its own variety of streptococcus, it will not necessarily be efficient against other varieties of the organism. And this he established by experimental evidence, thus elucidating the probable source of the great disagreement among observers as to the value of antistreptococcic sera. But he does not appear to have applied the same conclusions to the case of antityphoid sera, though, as Gruber and Durham have pointed out, the *B. typhosus* also exists in many varieties, exhibiting marked differences, which they denote as "special" in preference to "specific." Van der Velde also, working on rabbits in Denys' laboratory, had pointed out the necessity for a polyvalent antistreptococcic serum. In the present investigation, horses and rabbits were employed for the preparation of sera, and these, when obtained, were tested by a determination of their relative agglutinating power. Observations were also made on the protective power of the horse sera for guinea-pigs.

I was only concerned with the prepared sera. The management of the horses was under the care of Dr. Krumbein, assistant to Professor Tavel, and he continued to treat them in connection with the present work. I regret that the details of the treatment are somewhat incomplete, but the earlier part of it extended over a very considerable period before the commencement of my stay in Bern. The following particulars were kindly communicated to me by Dr. Krumbein :—

Two horses were employed, and at first filtered cultures were used for injection. Subsequently, cultures to which $\frac{1}{2}$ per cent. of carbolic acid had been added were employed. They were prepared in the following manner. A culture of the organism used (the variety denominated "F" below) was made in $1\frac{1}{2}$ litres of ordinary bouillon, and allowed to grow for a fortnight, at a temperature of 37° C. To this $\frac{1}{2}$ per cent. of carbolic acid was then added, and the fluid used for injection at once, and subsequently at appropriate intervals, until exhausted. A second culture, similarly prepared, was then begun, and so on. The injections were made subcutaneously, and were repeated so soon as all local and general reaction to the preceding injection had disappeared. They were increased gradually and very slowly until, after many months of treatment, a dose of 150 c.c. was reached at the end of 1898. The cultures do not appear to have been examined after the addition of carbolic acid before injection, to determine whether living bacilli remained. I am therefore unable to make any statement on this point. From time to time the horses suffered from abscesses at the seats of inoculation.

1. At the end of 1898, a dose of 150 c.c. of carbolised culture having been reached, blood was taken and serum prepared. This was examined in the laboratory in dilutions up to 1 in 10,000, and found to give a rapid agglutinative reaction.

2. The horses remained under treatment, and the injections were continued as before, the dose remaining constant at 150 c.c.

When the present investigation was begun in November 1899, Professor Tavel arranged for the treatment of the horses with mixed cultures. Accordingly, blood was again taken and serum prepared in December 1899, and injections then commenced of a mixture of cultures of the varieties F, G, S, T, below. The cultures were prepared as in the previous immunisations, and were mixed in about equal quantities for injection.

3. Beginning with small doses, the injection was gradually increased until, in March 1900, a maximum dose of 150 c.c. of the mixed carbolised cultures was attained. Serum was then again prepared.

4. Treatment was then begun with living cultures.

These were the horse sera with which I worked. I have named them serum 1898, serum 1899, and serum 1900 respectively, for the purposes of reference. The serum 1898 was a year old when examined, it had been kept in sealed tubes in the dark, and at a temperature but little above the freezing point during that period. The other sera were examined as obtained.

THE AGGLUTINATIVE REACTION.

The agglutinating power of the sera was determined on the six varieties of the *B. typhosus* available for the purpose. These were—

1.	Obtained from	Funck	and referred to as	F.
2.	"	do.	"	" G.
3.	"	Kral	"	" K.
4.	"	the Zürich		
		laboratory	"	" Z.
5.	"	Schottelius	"	" S.
6.	"	do.	"	" T.

F, then, was the variety used in the first two stages of the immunisation; and F, G, S, T those used in the third stage.

My stock cultures of the organisms were grown on glycerin agar, and were renewed about every three days throughout the investigation, in order to preserve their activity. From these cultures, subcultures for testing the agglutinative reaction were made in Dunham's solution—

Peptone	.	.	.	20 grms.
Sodium chloride	.	.	5 "	
Water	.	.	1000 c.c.	

prepared so as to be just alkaline to phenolphthalein. They were not used of longer than twelve to eighteen hours' growth.

The essential features of the agglutinative reaction were first observed by Pfeiffer, its correct interpretation was due to Gruber and Durham. The observation that the serum of typhoid patients has an agglutinating action on typhoid bacilli was first made by Grünbaum, working at the suggestion of Gruber, and to him is due the ordinary hanging-drop method of following the reaction. Widal was also an early worker in this field, and preceded Grünbaum in the publication of the method of serum diagnosis, which has consequently often been ascribed to him.

In the present investigation the method of obtaining the reaction ultimately adopted, and employed throughout the observations, was a modification of Wright's sero-sedimentation test, using small test-tubes, as described by Durham. The tubes used were about 5 or 6 mm. in diameter, and were carefully cleaned and sterilised before each determination. The cultures used were young cultures in Dunham's solution, usually from six to twelve hours old, but only about four hours old in the case of F, which exhibited a strong tendency to rapid self-agglutination, when grown in this solution.

Measurement was by dropping from a bulbed pipette. This is much simpler and probably more accurate as well as quicker than by graduations. With a graduated pipette much calculation and labour are required to obtain the necessary serum dilution, while this is a very simple matter by the method of counting drops. Further, a graduated pipette may be inaccurate, or become so if chipped at the point, and it is less easy to replace when broken, than one which can readily be made in a blowpipe whenever required from an ordinary piece of glass tubing. In dropping from a pipette, the size of the drop varies somewhat with the rate of flow, but this is easily regulated in practice; and the difficulty can be entirely obviated by drawing out the point of the pipette and modifying the calibre of the air inlet part above the bulb in such a way that the fluid will only drop, not run, even when the finger is entirely removed from the upper end. In such a case, with the upper opening left free, the rate of dropping is practically constant, and the drop produced of approximately constant volume. The dilutions were made as follows. Serum dilutions of the strength required were prepared in sterile plugged test tubes. These were put up fresh at least every three days, the serum being diluted with sterile distilled water. Thus, a hundred drops of water and one drop of serum gives a serum dilution of 1 to 100, one drop of this and fifty of water give a dilution of 1 to 5000, or one drop and a hundred of water a dilution of 1 to 10,000, and so on.

Supposing, then, that we desire to examine the agglutination at a dilution of 1 to 500,000, we obtain this dilution by measuring out a hundred drops of typhoid culture, and adding one drop of a 1 to 5000 serum dilution. If it is desired to examine the reaction at dilutions of 1 to 100,000, 110,000, and 120,000 respectively, we measure out into three tubes 100, 110, and 120 drops of typhoid culture; the pipette is then washed out several times with sterile distilled water, and dried and sterilised by heat; serum diluted to 1 to 1000 is then taken up into the pipette, and one drop allowed to fall into each of the tubes, and thoroughly mixed by gentle agitation. The pipette is carefully washed free from serum, and dried as before. By this method a large number of dilutions can rapidly be prepared. The tubes containing them are then fired at the mouth, plugged with cotton, and placed in the hot chamber until the reaction is complete. Obviously the same pipette must be used for both serum and culture; with this condition a drop of the diluted serum is for all practical purposes of the same size as a drop of culture.

The sterilisation of the tubes in which the reaction is made, before use, and the plugging of their mouths, is necessary in order to avoid contamination, which would seriously endanger the accuracy of the results obtained where the fluid is incubated for several hours in the hot chamber.

The reaction is very clear and definite, the opaque culture fluid becomes transparent, and is filled with clumps visible to the naked eye, as larger or smaller granules, which slowly settle to the foot of the tube. A control tube, without serum, was invariably put up at the same time as the serum dilutions; in this the culture remains turbid and opaque. When a reaction had been obtained the results were noted, and the tubes submitted to Professor Tavel for confirmation; and when the limits of agglutination appear to have been determined, the critical dilutions were repeated for further confirmation. In any case of doubt, hanging-drops were prepared from the tubes, in which the reaction appeared doubtful, and the microscope employed as a final control. The time required in the hot chamber for the reaction to be completed was usually about four hours in the case of F, about six hours in the case of Z, and from six to twelve hours with the other varieties.

The limit fixed throughout was that of Wright, namely, the highest dilution at which any reaction could be shown to occur. This criterion was chosen in preference to Durham's criterion, of the least percentage of serum which will completely precipitate a given amount of bacilli at a given temperature in a given time, because it was clearly desirable to use the same method of determination throughout the whole of the experiments, and it could not be known beforehand whether the sera to be prepared from rabbits would have reached a sufficient degree of potency to give "complete reaction" at dilutions high enough and differing sufficiently among themselves to supply the necessary differentiation between the various typhoid organisms employed. No attempt was made to obtain nearer approximations to the limit of agglutinative action than about 5 to 10 per cent. of the total dilution. This was found to be sufficient to differentiate the varieties used.

Unavoidable errors from unequal size of drops in pipetting, evaporation in the hot chamber affecting dilution, personal equation, and so on, may probably amount to as much as 1 per cent. of the total dilution. It is true that Winterberg, recently, dealing with the effect on the agglutinating power of a serum of growing various bacteria in that serum, records differences of as little as 5 in 1800 or more, *i.e.* only about $\frac{1}{4}$ per cent. of the whole dilution. But I should imagine such differences to come well within the limits of experimental error, whatever be the method employed in determination.

As an example of the manner in which the test was applied and the results recorded, the following may be chosen from many hundreds of observations:—

SERUM 1899.

Bacillus K.

+ = positive reaction, — = no reaction.

Tube.	Dilution.	Drops of Culture.	Drops of Serum diluted to 1 to 5000.	Result.
1	1 to 450,000	90	1	+
2	„ 500,000	100	1	+
3	„ 550,000	110	1	+
4	„ 600,000	120	1	—
5	„ 650,000	130	1	—
6	„ 700,000	140	1	—
7	Control	100	Nil	—

RABBITS' SERUM.

Series C. Stage II.

Bacillus Z.

+ = positive reaction, -- = no reaction.

Tube.	Dilution.	Drops of Culture.	Drops of Serum diluted to 1 to 100.	Result.
1	1 to 5,000	100	2	+
2	" 6,000	120	2	+
3	" 7,000	140	2	+
4	" 8,000	80	1	+
5	" 9,000	90	1	+
6	" 10,000	100	1	-
7	" 11,000	110	1	-
8	Control	100	Nil	-

AGGLUTINATION BY IMMUNE SERA OF HORSE.

The agglutinative limits obtained with the horse sera are tabulated on p. 257. The sign + indicates "positive reaction" the sign - that no reaction occurred. They appear in the form of a chart in the accompanying diagram.

Table of Agglutination by Horse Sera.

+ = positive reaction, - = no reaction,

Bacillus.	Serum 1898.	Serum 1899.	Serum 1900.
F . . . {	1 to 280,000 +	1 to 2,500,000 +	1 to 2,500,000 +
	,, 300,000 -	,, 3,000,000 -	,, 3,000,000 -
Z . . . {	,, 100,000 +	,, 650,000 +	,, 650,000 +
	,, 110,000 -	,, 700,000 -	,, 700,000 -
S . . . {	,, 90,000 +	,, 600,000 +	,, 1,100,000 +
	,, 100,000 -	,, 650,000 -	,, 1,200,000 -
K . . . {	,, 80,000 +	,, 550,000 +	,, 550,000 +
	,, 90,000 -	,, 600,000 -	,, 600,000 -
T . . . {	,, 75,000 +	,, 400,000 +	,, 650,000 +
	,, 80,000 -	,, 450,000 -	,, 700,000 -
G . . . {	,, 70,000 +	,, 400,000 +	,, 550,000 +
	,, 75,000 -	,, 450,000 -	,, 600,000 -

Observing these results the following points may be noted :—

1. The action of an antityphoid serum is very much more marked upon the variety of the bacillus by means of which it has been produced than upon other varieties of the organism.

2. Such other varieties can be differentiated by the degree of agglutinative action which the serum has upon them; that is to say, the serum has a "special" action on its own organism, and a non-special action varying in degree on other varieties of the organism. The fact that such "special" differences exist between the varieties of an organism and between their sera was first noted by Gruber and Durham *in vitro*, both in the case of typhoid and in that of cholera. It has been confirmed by Pfeiffer and Kolle *in vivo*. Durham has also pointed out that the persistence of the serum reaction against a given bacillus, after it has been lost against allied bacilli, is in favour of the original infection having been due to that bacillus.

3. On comparing the results of serum 1898 with those of serum 1899, we see that while the dose used in the inoculation throughout the year remained constant at the maximum reached in 1898, before the first preparation of serum, the agglutinative power has increased very greatly, though allowance must probably be made for some deterioration of the 1898 serum by keeping.

4. The increase, moreover, has been much more rapid for the immunising bacillus than for the other varieties, being between nine and ten-fold for the former, but only about six or seven-fold for the latter; that is to say, that, as immunity increases, the "specialism" of the serum for its own bacillus increases. It becomes more specialised.

5 As might be expected, the relative action of the serum on other varieties of the organism is found to remain unchanged; that is to say, that, when tabulated according to the degree of agglutinative dilution obtained, they fall into the same order of succession.

6. If now we consider serum 1900, for the production of which

the varieties F, G, S, T had been used, we see, first of all, that although the dose of F had been reduced, so as not to exceed one-fourth of its former amount—for the maximum dose of the mixed cultures was only equal to the dose of F alone in the 1899 immunisation—yet the agglutinative power of the serum toward F has not diminished to any marked extent, for it still lies between the limits (1 to 2½ millions positive reaction, 1 to 3 millions no reaction) previously obtained. In other words, the degree of “special” agglutinative power obtained does not tend to diminish with any great rapidity.

7. Similarly, the non-special agglutinative power on other varieties of the organism is unchanged:—compare the action on the bacilli Z and K.

8. In the case of G, S, and T, there has been a marked increase in agglutinating action, the serum having become “special” for these three varieties also, by reason of their progressive inoculation in the horses. And this increase, we observe, has been unequal in the different cases, being greater for those varieties previously most acted upon by the serum which was “special” only for F.

9. It is to be noted also, as indicating the value and importance of polyvalent sera, that though the maximum dose of S, for example, was only about one quarter of 150 c.c., and the treatment of only three months duration, yet the serum so produced is much more efficient against S than the serum obtained in 1899, after dosage with 150 c.c. of another variety, F, had continued for the whole year, following a prolonged treatment with smaller doses of the same bacillus.

10. It appears, also, that equal immunising doses of different varieties do not produce equal agglutinating powers of serum, for while S is agglutinated by dilutions of 1 to 1,100,000, the highest dilution obtained for T is 1 to 650,000, and for G only 1 to 550,000.

We shall have to return later to the discussion of some points illustrated by the agglutinating actions of these sera. We must now turn for a little to a consideration of the sera prepared from rabbits.

IMMUNISATION OF RABBITS.

Three of the varieties of typhoid were chosen for this work—F, Z, K, and three series of rabbits were employed, two in each series. The plan carried out was as follows:—

Let us call the series of rabbits A, B, and C; the organisms were F, Z, K. Each series was partly immunised by repeated increasing injections of one of the bacilli (A by F, B by K, and C by Z). Blood was then taken and serum prepared. The bacilli were then interchanged between the series, and the rabbits again injected up to the same maximal doses as before; Series A being now treated with Z, B with F, and C with K. Serum was then again prepared. The procedure was now repeated a third time (A being treated on this occasion

with K, B with Z, and C with F), and serum obtained. Finally, one rabbit from Series A and one from Series C received, at a short interval after the completion of the third period of inoculation, their maximum doses of the two preceding inoculations, *i.e.* for A the maximum doses first of Z and then of F, and for C that of K and then of Z.

This scheme of immunisation may be tabulated as follows, dates being inserted :—

Scheme of Immunisation of Rabbits.

		SERIES A. Rabbits 1 and 2.	SERIES B. Rabbits 1 and 2.	SERIES C. Rabbits 1 and 2.
Stage I.	Inoculated from Nov. 11 to Dec. 12.	With increasing doses of F.	With increasing doses of K.	With increasing doses of Z.
	Blood taken Dec. 20.	From A 2.	From B 1.	From C 1.
Stage II.	Inoculated from Jan. 9 to Jan. 31.	With increasing doses of Z.	With increasing doses of F.	With increasing doses of K.
	Blood taken Feb. 7.	From A 2.	From B 1.	From C 1.
Stage III.	Inoculated from Feb. 12 to Feb. 27.	With increasing doses of K.	With increasing doses of Z.	With increasing doses of F.
	Blood taken Mar. 5.	From A 2.	From B 1.	From C 1.
Stage IV.	Inoculated Mar 6.	A 1 <i>only</i> . With max. dose of Z.	...	C 2 <i>only</i> . With max. dose of K.
	" Mar 8.	With max. dose of F.	...	With max. dose of Z.
	Blood taken Mar. 13.	From A 1.	...	From C 2.

The animals were treated with gradually increasing doses, their temperatures were taken almost every day, and the weights three or four times a week. The weight appeared, in practice, to afford a better indication of the condition of the animal than its temperature. All the animals were always injected at the same time, and the same series of doses was used in the three successive immunisations except that the process was successively shortened in the second and third periods by omitting the initial smaller doses of the series. The fourth period consisted simply of injecting, in the two animals chosen (A 1 and C 2), the maximum doses which had been used in the second and first period respectively. The animals were of the same age, and the weights of those from which serum was prepared were not at any time widely different. The cultures injected were young cultures¹ in Dunham's

¹ Twenty-four hours old.

solution. In the earlier injections these had been killed by being kept for half an hour at a temperature of 58° C.; in the later stages living cultures were used. The injections were made subcutaneously. One animal (B 2) suffered from abscess. This was not in the neighbourhood of the points of inoculation. The serum of this animal was not used.

After the injections were completed, the animals were left for a week or more before bleeding, and were subsequently allowed time to recover from the bleeding before treatment was recommenced.

The weights of the animals increased steadily throughout the period of observation.¹

Blood was taken from the external jugular vein, except in the case of B 1 and C 1, where the femoral artery was employed at the third operation. The method found most satisfactory was by means of a sharp-pointed cannula, inserted through the wall of the exposed vessel. A relatively large cannula was used, provided at its distal end with a short piece of rubber tubing, by which the blood was conveyed into the receiver. By this means a test tube full of blood was readily obtained. The clot was loosened, after about half an hour, and the tube placed in the cold chamber. The serum resulting was drawn off on the following day into a number of small tubes, and sealed up until required for use. All apparatus was of course carefully sterilised, and the operation wounds healed aseptically in every case. The sera were tested, as obtained by the agglutinative reaction, against the three typhoid bacilli, F, K, Z, and also against the variety T. The results obtained are tabulated below, the sign + standing for positive reaction, and — for no reaction, as in the previous case.

AGGLUTINATION BY IMMUNE SERA OF RABBITS.

Table of Agglutination by Rabbits' Sera.

Imm. = Immunisation.

TABLE I. Bacillus I.	SERIES A.	SERIES B.	SERIES C.
	Imm. with F. Serum gives—	Imm. with K. Serum gives—	Imm. with Z. Serum gives—
F . . . {	1 to 11,000 + ,, 12,000 —	1 to 3500 + ,, 4000 —	1 to 5,000 + ,, 6,000 —
K . . . {	,, 3,500 + ,, 4,000 —	,, 6500 + ,, 7000 —	,, 4,000 + ,, 4,500 —
Z . . . {	,, 6,000 + ,, 7,000 —	,, 3000 + ,, 3500 —	,, 10,000 + ,, 11,000 —
T . . . {	,, 3,000 + ,, 3,500 —	,, 1500 + ,, 2000 —	,, 3,500 + ,, 4,000 —

¹ The detailed tables of the injections, weights, and temperatures, extending over more than four months, are omitted, from considerations of space.

Table of Agglutination by Rabbits' Sera—continued.

TABLE II. Bacillus II.	SERIES A.	SERIES B.	SERIES C.
	Imm. with F, followed by imm. with Z. Serum gives—	Imm. with K, followed by imm. with F. Serum gives—	Imm. with Z, followed by imm. with K. Serum gives—
F . . . {	1 to 9,000 + ,, 10,000 -	1 to 11,000 + ,, 12,000 -	1 to 4,500 + ,, 5,000 -
K . . . {	,, 4,000 + ,, 4,500 -	,, 6,000 + ,, 6,500 -	,, 6,500 + ,, 7,000 -
Z . . . {	,, 10,000 + ,, 11,000 -	,, 6,000 + ,, 7,000 -	,, 9,000 + ,, 10,000 -
T . . . {	,, 3,500 + ,, 4,000 -	,, 3,000 + ,, 3,500 -	,, 3,500 + ,, 4,000 -

TABLE III. Bacillus III.	Imm. with F, followed by imm. with Z, followed by imm. with K. Serum gives—	Imm. with K, followed by imm. with F, followed by imm. with Z. Serum gives—	Imm. with Z, followed by imm. with K, followed by imm. with F Serum gives—
	Serum gives—	Serum gives—	Serum gives—
F . . . {	1 to 9,000 + ,, 10,000 -	1 to 10,000 - ,, 11,000 -	1 to 11,000 + ,, 12,000 -
K . . . {	,, 6,500 + ,, 7,000 -	,, 6,000 + ,, 6,500 -	,, 6,000 + ,, 6,500 -
Z . . . {	,, 10,000 + ,, 11,000 -	,, 10,000 + ,, 11,000 -	,, 9,000 + ,, 11,000 -
T . . . {	,, 3,000 + ,, 3,500 -	,, 3,500 + ,, 4,000 -	,, 3,500 + ,, 4,000 -

TABLE IV. Bacillus IV.	Max. doses of Z and F, repeated after Stage III. Serum gives—	Max. doses of K and Z, repeated after Stage III. Serum gives—
	Serum gives—	Serum gives—
F . . . {	1 to 11,000 + ,, 12,000 -	1 to 11,000 + ,, 12,000 -
K . . . {	,, 6,500 + ,, 7,000 -	,, 6,500 + ,, 7,000 -
Z . . . {	,, 10,000 + ,, 11,000 -	,, 10,000 + ,, 11,000 -
T . . . {	,, 3,500 + ,, 4,000 -	,, 3,500 + ,, 4,000 -

1. Taking Table I. we see again that, as in the case of horse sera, 1898 and 1899, each serum is special for its own bacillus.

2. Further, this "special" action varies for the different series, although the same immunising doses were used in each case; that is to say, the same dosage with different varieties of typhoid produces not the same, but different degrees of agglutinative efficiency against

those varieties. This was also noted in the case of the horse, where the doses of the different organisms had been injected together into the same animal (serum 1900).

3. Again, comparing the tables, it becomes clear that previous immunisation with a particular variety of typhoid, as F, although producing a serum which has a considerable agglutinative action on another variety, say Z, does not enable that variety, when itself used in a second period of immunisation, to produce a serum of higher "special" agglutinative power for Z than is obtained by immunisation with Z alone. And this is seen to hold throughout. In other words, the "special" agglutinative action of a serum on its own bacillus, obtained after the injection of certain doses of that bacillus, is not increased by previous or subsequent treatment of the animal with corresponding doses of other varieties of the bacillus. There is, therefore, no cumulative action or addition of agglutinating powers of the constituent monovalent sera in the production of a polyvalent serum. That is to say, the valency of polyvalent sera against any one of the varieties of typhoid used in its production is neither greater nor less than that of the monovalent serum obtained by the use of that variety alone.

4. Further, the polyvalent serum has no more powerful an action on an independent variety of the bacillus than the most powerful toward that bacillus of its monovalent constituents. Thus, of the monovalent sera in Table I., the most efficient against T is that produced by immunisation with Z, and this degree of efficiency is not surpassed in Table III. These facts appear to be of some importance in relation to the question of the nature of agglutination, and of those "bodies," or probably more correctly those physico-chemical conditions, in the serum known as "agglutinins,"—for no one has as yet been able to separate an agglutinin from serum as a physical entity.

5. Comparing Tables I. and II., Series A, we note that while the monovalent serum with F, agglutinated F up to a dilution between 1 to 11,000 and 1 to 12,000, the duovalent serum of F and Z only gives a reaction with F up to a dilution between 1 to 9000 and 1 to 10,000. Apparently, then, during the period intervening between the end of the immunisation with F, and the completion of that with Z, the efficiency of the serum against F has diminished. On turning to Table III. no further diminution is exhibited. This is probably to be explained, on the supposition that the diminution occurring falls within the limits taken; and such a supposition is quite admissible, since it is easily possible that in Table I. the absolute limit of agglutination is not more than one or two hundreds, say, above the dilution of 1 to 11,000, and the period of time during which diminution is going on, namely, from the end of the first stage of immunisation to the completion of the second stage, was, owing to the intervention of

the Christmas vacation, nearly twice as long as the second period of diminution, namely, from the end of the second stage to the completion of the third (compare F in Series B, Tables II. and III.).

If we examine the reactions of Z in Series C, the same phenomena are observed, the monovalent serum acts up to limits between 1 to 10,000 and 1 to 11,000, the duovalent to limits between 1 to 9000 and 1 to 10,000, and the trivalent to limits between 1 to 9000 and 1 to 10,000 also. And similarly with K in Series B. The "special" agglutinative power already obtained toward one variety of typhoid is, therefore, found to diminish when the injections are discontinued, in spite of immunisation with another variety of the bacillus being in progress.

6. If we compare Tables II. and III., Series A, we shall see that the non-special agglutinative power of the duovalent serum in Table II. toward T also diminishes during the period of immunisation of the animals in this series with K, and we shall correlate this observation with the fact that the action of K serum on T is much less than that of F serum or Z serum on the same organism.

SERIES A.

7. That these partial losses of agglutinative efficiency are readily restored is shown by Table IV., where the animals had received a single maximal dose of each of the bacilli used, in comparatively rapid succession, before bleeding. Here the sera are at their maximum efficiency against all the organisms, having the "special" power of each

of the monovalent sera and a non-special efficiency against T, equal to that of the most efficient monovalent serum. Series B does not appear

SERIES B.

in this table, owing to the fact that rabbit 1 had been killed, and rabbit 2 was only just recovering from an abscess, and was very much out of condition, and therefore not used for serum preparation.

On the whole, then, it appears that—as regards agglutinating power, at any rate—a serum is valent against a given variety of the typhoid bacillus, in proportion

as it is “special” to that variety, and that therefore the most generally efficient serum against the typhoid bacillus in general will be the one which is most polyvalent.

Now, Durham has brought forward considerable evidence of a correlation between agglutinative power and protective power. But, if such a relation actually exists, it follows, from the foregoing observations, that

SERIES C.

the most polyvalent serum will also be the most generally protective, and that therefore one of the first essentials for the production of a serum of therapeutic value is, that it shall be made extensively polyvalent. To this point, however, we shall return later. The accompanying charts supply a diagrammatic representation of the successive changes in the rabbits' sera.

NATURAL SEDIMENTATION, AGGLUTINATION, AND VIRULENCE.

Reference has already been made to a very marked tendency exhibited by the bacillus F to early sedimentation, when growing in Dunham's solution. The same tendency was exhibited, in greater or less degree, by all the varieties of typhoid bacilli under observation. I therefore proceeded to determine approximately the relative rapidity with which this sedimentation occurred. For this purpose tubes containing about 10 c.c. of Dunham's solution were inoculated with one small loopful of each of the bacilli. The tubes were placed in the incubator and examined each hour. The results of a number of observations were as follows:—

Table of Commencement of Natural Sedimentation.

With F	Sediment detected in time varying from 1 to 3 hours.			Average of all observations, 2 hours.		
" Z	" "	" 3 "	5 "	" "	" 4 "	
" S	" "	" 10 "	15 "	" "	" 12 "	
" K	" "	" 16 "	24 "	" "	" 20 "	
" T	" "	" 20 "	28 "	" "	" 24 "	
" G	" "	" 24 "	36 "	" "	" 30 "	

It is at once observed that the bacilli Z, S, K, T, G assume in this series the order of their respective reaction to agglutination by serum 1898 and serum 1899, and so far as Z, K, and T are concerned, the order of their agglutination by the monovalent F serum of the rabbit (Series A, Table I.); and that F, which produces in the rabbit a monovalent serum of higher "special" power than that produced by either Z or K (being agglutinated by its serum in dilutions up to between 1 to 11,000 and 1 to 12,000, as against dilutions of between 1 to 10,000 and 1 to 11,000, and 1 to 6500 and 1 to 7000 for Z and K respectively), is the most prone to this natural sedimentation, if we may so term it. It would seem, therefore, at first sight, that great vegetative activity is associated with susceptibility to agglutination; for the sedimentation appeared to begin when the cultures reached a certain degree of opacity.

Wyatt Johnston has stated that "active virulent cultures" tend to give this natural sedimentation, while old attenuated cultures do not, when similarly inoculated in a fluid medium. The question arises, therefore, whether the vegetative "activity" is separable from the "virulence" in this proposition, and if so, whether it is the activity

or the virulence which is associated with the susceptibility referred to. It was accordingly desirable to determine the respective virulence of the various bacilli, and a series of observations were made on guinea-pigs for this purpose. At first, cultures in Dunham's solution were injected, but these not succeeding, in any dose of reasonable volume, agar cultures of forty-eight-hours' growth were subsequently employed. The growth was carefully washed off the sloped surface with a measured quantity of sterile Dunham's solution, and the necessary amount, *e.g.* $\frac{1}{4}$, $\frac{1}{3}$, etc., injected into the peritoneal cavity. Twenty-four hours was the limit of time taken within which the animal must die, and the following were the series of observations made:—

With F.

Guinea-pig.	Weight in Grms.	Total Dose (Intraperitoneal).	Dose per 100 Grms. Weight.	Result in 24 Hours.	Result Later.
1	385	5 c.c. of a 24-hour culture in Dunham's solution.	1.3 c.c. of a 24-hour culture.	Nil.	Nil.
2	400	10 c.c. " " "	2.5 c.c. " "	"	"
3	435	6 c.c. of a 48-hour culture in Dunham's solution.	1.38 c.c. of a 48-hour culture.	"	"
4	555	12 c.c. " " "	2.16 c.c. " "	"	"
5	500	(1) 48-hour agar culture.	0.2 c.c. of a 48-hour agar culture.	"	"
6	650	(2) " " cultures	0.3 c.c. " "	"	"
7	560	(3) " " "	0.5 c.c. " "	"	"
8	365	(4) " " "	1.1 c.c. " "	"	"
9	220	(5) " " "	2.27 c.c. " "	Died in 20 hours.	...

Minimum dose per 100 grms. weight, fatal within 24 hours, lies between 1.1 and 2.27 of a 48-hour agar culture.

With T.

Guinea-pig.	Weight in Grms.	Total Dose (Injected Intraperitoneally).	Dose per 100 Grms. Weight.	Result in 24 Hours.	Result Later.
1	575	1 c.c. of a 24-hour culture in Dunham's solution.	0.19 c.c. of a 24-hour culture.	Nil.	Nil.
2	550	3 c.c. " " "	0.54 c.c. " "	"	"
3	540	3 c.c. of a 48-hour culture in Dunham's solution.	0.5 c.c. of a 48-hour culture.	"	"
4	600	6 c.c. " " "	1 c.c. " "	"	"
5	525	$\frac{1}{3}$ of a 48-hour agar culture.	0.06 c.c. of a 48-hour agar culture.	"	"
6	705	$\frac{1}{3}$ " " " "	0.07 c.c. " "	"	"
7	600	$\frac{1}{3}$ " " " "	0.083 c.c. " "	"	"
8	680	1 " " " "	0.147 c.c. " "	Died within 20 hours.	...
9	500	$\frac{1}{2}$ " " " "	0.1 c.c.	Died within 23 hours.	...

Minimum dose per 100 grms. weight, fatal within 24 hours, lies between 0.083 and 0.1 of a 48-hour agar culture.

With K.

Guinea-pig.	Weight in Grms.	Total Dose (Injected Intra-peritoneally).	Dose per 100 Grms. Weight.	Result in 24 Hours.	Result Later.
1	500	$\frac{1}{2}$ of a 48-hour agar culture.	0.1 c.c. of a 48-hour agar culture.	Nil.	Nil.
2	495	$\frac{5}{8}$ „ „ „ „	0.126 c.c. „	„	„
3	705	1 „ „ „ „	0.142 c.c. „	Ill.	Died in 30 hours.
4	565	1 „ „ „ „	0.177 c.c. „	Died in 20 hours.	...

Minimum dose per 100 grms. weight, fatal within 24 hours, lies between 0.142 and 0.177 of a 48-hour agar culture.

With Z.

Guinea-pig.	Weight in Grms.	Total Dose (Injected Intra-peritoneally).	Dose per 100 Grms. Weight.	Result in 24 Hours.	Result Later.
1	670	1 48-hour agar culture.	0.149 c.c. of a 48-hour agar culture.	Nil.	Nil.
2	650	1 „ „ „	0.153 c.c. „	Ill.	Recovered.
3	490	1 „ „ „	0.204 c.c. „	„	Died in 28 hours.
4	430	1 „ „ „	0.232 c.c. „	Died within 22 hours.	...

Minimum dose per 100 grms. weight, fatal within 24 hours, lies between 0.204 and 0.232 of a 48-hour agar culture.

With S.

Guinea-pig.	Weight in Grms.	Total Dose (Injected Intra-peritoneally).	Dose per 100 Grms. Weight.	Result in 24 Hours.	Result Later.
1	380	$\frac{1}{2}$ of a 48-hour agar culture.	0.131 c.c. of a 48-hour agar culture.	Nil.	Nil.
2	390	$\frac{3}{8}$ „ „ „ „	0.154 c.c. „	„	„
3	390	$\frac{3}{4}$ „ „ „ „	0.192 c.c. „	Ill.	Recovered.
4	370	$\frac{3}{4}$ „ „ „ „	0.202 c.c. „	Died in 20 hours.	...

Minimum dose per 100 grms. weight, fatal within 24 hours, lies between 0.192 and 0.202 of a 48-hour agar culture.

With G.

Guinea-pig.	Weight in Grms.	Total Dose (Injected Intra-peritoneally).	Dose per 100 Grms. Weight.	Result in 24 Hours.	Result Later.
1	670	1 48-hour agar culture.	0.15 c.c. of a 48-hour agar culture.	D i e d within 16 hours.	...
2	665	$\frac{7}{10}$ " " "	0.105 c.c. "	Died in 16 hours.	...
3	410	$\frac{3}{10}$ " " "	0.073 c.c. "	Died in 20 hours.	...
4	400	$\frac{7}{10}$ " " "	0.075 c.c. "	Died in 16 to 20 hours.	...
5	500	$\frac{3}{10}$ " " "	0.03 c.c. "	Nil.	Nil.
6	425	$\frac{1}{10}$ " " "	0.047 c.c. "	Ill.	D i e d within 30 hours.

Minimum dose per 100 grms. weight, fatal within 24 hours, lies between 0.047 and 0.073 of a 48-hour Agar Culture.

The minimum doses per 100 grms., of guinea-pig, fatal within twenty-four hours, are therefore—

For G, between 0.047 and 0.073 of a 48-hour agar culture.

" T,	"	0.083	"	0.1	"	"	"	"
" K,	"	0.142	"	0.177	"	"	"	"
" S,	"	0.192	"	0.202	"	"	"	"
" Z,	"	0.204	"	0.232	"	"	"	"
" F,	"	1.1	"	2.27	"	"	"	"

and this, therefore, the order of virulence of these bacilli. It is also the exact inverse of the order of susceptibility to agglutination and of tendency to natural sedimentation.

1. We may therefore conclude, at least so far as the evidence of the six varieties of typhoid observed goes, that, of a series of typhoid varieties examined, those showing a greater tendency to natural sedimentation will be more susceptible to agglutination by a given antityphoid serum, not "special" to any one of them, and also less virulent than those in which this tendency is less pronounced.

2. As regards agglutination and virulence, it appears that the susceptibility to agglutination is in inverse relation to the respective virulence of a series of the bacilli, tested against a serum which is "special" to none of them. This proposition is borne out by the results recorded both with the horse sera and with the rabbits. Thus, the monovalent rabbits' serum of Series B, "special" only for K, agglutinates F Z T as follows:—

F,	1 to 3500 +,	1 to 4000 -,
Z,	1 " 3000 +,	1 " 3500 -,
T,	1 " 1500 +,	1 " 2000 -,

that is to say, in the inverse order of their virulence.

Durham states that, in the case of sixteen different vibrios which he has examined, the less virulent races were more susceptible to the agglutinins produced by the use of virulent varieties than were those virulent varieties themselves. Moreover, the serum obtained by means of the less virulent, though highly effective on them, had slight influence on the more virulent. This observation apparently does not hold in the case of typhoid bacilli, for, as we have seen in the case of all the monovalent sera examined, a given serum is always most active against its own variety of the bacillus, irrespective of virulence, and that the sera prepared with less virulent organisms—notably the horse sera 1898 and 1899, prepared by the use of the but slightly virulent variety F—have very definite influence on the more virulent races also. And this accords with Durham's own statement with regard to this group of organisms, that persistence of reaction against a given bacillus, after it has been lost against allied bacilli, is in favour of the original infection having been due to that bacillus. For the reaction will evidently persist longest for that variety for which it was originally most marked.

It would have been of considerable interest to determine whether, and if so in what manner, the agglutinative reaction of a particular bacillus of low virulence, such as F, to a serum "special" for F, was affected when the virulence of F was progressively increased by passage. For, if the view above is correct, the limit of dilution at which reaction occurred should be progressively lowered. Some attempt was made in this direction, but I was unfortunately unable, in the brief time remaining at my disposal at this period, and with the very few animals available, to pursue the subject to a satisfactory conclusion. The very great difficulty experienced in obtaining a suitable supply of guinea-pigs also explains the considerable irregularity noticeable in the weights of the animals used in the observations on the comparative virulence of the different races of typhoid employed.

PROTECTIVE POWER OF HORSE SERA.

I was at this time engaged in endeavouring to determine the protective power of the horse sera, and the results obtained are tabulated below. They are not complete, having necessarily terminated when no further supply of suitable animals could be procured.

In the experiments for determining protective power, serum and culture were mixed, and injected together into the peritoneal cavity. A control experiment, without serum, was made at the same time in each case. The observations were as follow:—

SERUM 1899.

TABLE V.—*Bacillus Z.*

Guinea-pig.	Weight in Grms.	Total Dose of Culture.	Dose per 100 Grms. Weight.	Dose of Serum.	Serum per 100 Grms. Weight.	Result.
1	430	1 of a 48-hour agar culture.	0·232 of a 48-hour agar culture.	1 c.c.	0·232 c.c.	Nil.
1A	440	1 " "	0·231 " "	Nil.	...	Died within 24 hours.
2	425	1 " "	0·235 " "	0·5 c.c.	0·117 c.c.	Ill; recovered.
2A	429	1 " "	0·233 " "	Nil.	...	Died within 24 hours.

Hence, 0·117 c.c. serum per 100 grms. weight protects.

TABLE VI.—*Bacillus K.*

Guinea-pig.	Weight in Grms.	Total Dose of Culture.	Dose per 100 Grms. Weight.	Dose of Serum.	Serum per 100 Grms. Weight.	Result.
1	415	$\frac{3}{4}$ of a 48-hour agar culture.	0·1807 of a 48-hour agar culture.	0·5 c.c.	0·1206 c.c.	Died within 24 hours.
1A	420	$\frac{3}{4}$ " "	0·178 " "	Nil.	...	Died in 20 hours.
2	400	$\frac{3}{4}$ " "	0·187 " "	1 c.c.	0·25 c.c.	Nil.
2A	405	$\frac{3}{4}$ " "	0·185 " "	Nil.	...	Died in 16–20 hours.
3	500	$\frac{1}{10}$ " "	0·18 " "	1 c.c.	0·2 c.c.	Off its feed; recovered.
3A	556	1 " "	0·179 " "	Nil.	...	Died within 24 hours.

Hence, 0·2 c.c. serum per 100 grms. weight protects.

TABLE VII.—*Bacillus T.*

Guinea-pig.	Weight in Grms.	Total Dose of Culture.	Dose per 100 Grms. Weight.	Dose of Serum.	Serum per 100 Grms. Weight.	Result.
1	480	$\frac{1}{2}$ of a 48-hour agar culture.	0·104 of a 48-hour agar culture.	1 c.c.	0·208 c.c.	Died within 24 hours.
1A	485	$\frac{1}{2}$ " "	0·103 " "	Nil.	...	Died in 20 hours.
2	505	$\frac{1}{2}$ " "	0·099 " "	1·25 c.c.	0·247 c.c.	Died within 24 hours.
2A	510	$\frac{1}{2}$ " "	0·098 " "	Nil.	...	Died within 18 hours.
3	500	$\frac{1}{2}$ " "	0·1 " "	1·5 c.c.	0·3 c.c.	Nil.
3A	505	$\frac{1}{2}$ " "	0·099 " "	Nil.	...	Died within 20 hours.

Hence, 0·3 c.c. serum per 100 grms. weight protects.

SERUM 1900.

TABLE VIII.—*Bacillus T.*
These three experiments were simultaneous.

Guinea-pig.	Weight in Grms.	Total Dose of Culture.	Dose per 100 Grms. Weight.	Dose of Serum.	Serum per 100 Grms. Weight.	Result.
1	516	$\frac{1}{2}$ of a 48-hour agar culture.	0·0969 of a 48-hour agar culture.	1 c.c.	0·1938 c.c.	Nil.
2	525	$\frac{1}{2}$ „ „	0·095 „ „	1·25 c.c.	0·238 c.c.	Nil.
3	533	$\frac{1}{2}$ „ „	0·0938 „ „	Nil.	...	Died in 20-22 hours.

Hence, 0·1938 c.c. serum per 100 grms. weight protects.

We see, then, from these tables that, representing the “minimum dose, per 100 grms. of guinea-pig, fatal within twenty-four hours,” by the sign M.L.D., in each case a dose equal to or a shade greater than the M.L.D. was given, and that the animals with serum received, if anything, a slightly greater dose than the control animals. And we find, from a comparison of Tables V., VI., VII., that, in the case of the 1899 serum—

For Z	0·117 c.c. of serum per 100 grms. weight	Protects against the M.L.D. of Z.
For K	0·1206 „ „	Does not protect against the M.L.D. of K.
	0·2 „ „	Protects against the M.L.D. of K.
For T	0·247 „ „	Does not protect against the M.L.D. of T.
	0·3 „ „	Protects against the M.L.D. of T.

and comparing the action of serum 1899 on T with that of serum 1900 on the same bacillus (Tables VII. and VIII.)—

For T	0·247 c.c. of serum 1899 per 100 grms. weight	Does not protect against the M.L.D. of T.
	0·1938 „ „ 1900 „ „	Protects against the M.L.D. of T.

And possibly a smaller dose than 0·1938 c.c. of the serum 1900 may be sufficient; but I was unable to make further trials, from lack of animals.

The horse sera, therefore, are protective for guinea-pigs against the bacilli Z, K, T, the dose required per 100 grms. being greater, with greater virulence of the variety employed. And horse serum

1900, which has been made "special" for T, is much more powerfully protective against that bacillus than is the serum 1899, which is not "special" for T. It follows, therefore, for protection also, just as for agglutination, that, in order to produce a serum having high efficiency against typhoid organisms in general, we must make that serum special for as many different varieties of the organism as possible.

RELATION OF AGGLUTINATION TO PROTECTION.

We have seen that as regards the three varieties, Z, K, T, the protective power of the serum used is in proportion, or, more correctly, in relation, to its agglutinating power on those organisms; and that, as regards the variety T, an increase in the protective power of the serum is associated with an increase in its agglutinative efficiency against T. This is in accord with Durham's contention that agglutination and protection are related phenomena. It is evident, however, from a comparison of the tables of agglutination with those of protection that this relation is not a simple one of proportion.

Many observers have denied the existence of any relation between agglutination and protection, on the ground that agglutinative properties are not essential to protection, and Widal is even of the opinion that high agglutinative power may be associated with very low protective power. Sims Woodhead also, while not agreeing with Widal's contention, has pointed out that the most highly protective sera—those of diphtheria and tetanus—are not agglutinative, and the highly agglutinative cholera and typhoid sera cannot be raised to such a degree of immunising activity. But, as already noted, typhoid and cholera sera differ fundamentally from pure antitoxines, such as are the diphtheria and tetanus sera. They belong to an entirely different category, and cannot properly be compared with them in this respect, for all recent work tends to confirm the position that essential differences exist in the nature of the processes concerned. In the case of diphtheria and tetanus, the general disease is purely toxic, and the poison is present in the form of a body having certain chemical affinities for its antitoxine, and rendered powerless by union with that antitoxine. There is, in fact, as Cobbett and others have shown, a direct action of antitoxine on toxine. In the terminology of Ehrlich, the poison molecule is composed of a toxic atom-complex (toxophore) and a chemically unsatisfied "anchoring" atom-complex (haptophore), by means of which the toxophore can be linked to cells, and come to act upon them; but which has even greater affinity for the antitoxine than for cells. The haptophore, therefore, rapidly links the poison molecule to the antitoxine, and produces thereby a chemically satisfied and physiologically inert molecular system. And this occurs equally, whether the experiment be made *in vivo* or *in vitro*. In

typhoid and cholera, however, we are concerned with antimicrobial sera, and the conditions appear to be entirely different. The contention that the relation suggested between agglutination and protection is non-existent is also strongly supported by Pfeiffer and Kolle, and Fränkel and Otto have argued in the same direction; but Durham has clearly demonstrated the inconclusiveness of Pfeiffer's experiments.

Now, it has been shown above, that an increase in agglutinating power of the serum of a given animal is accompanied by an increase in the protective power of that serum (compare serum 1899 and serum 1900 in relation to bacillus T.) And Durham has proved that "complete" agglutinative power, complete lysogenetic power, and complete protection are associated; while Gruber found that if the agglutinative power of a serum be diminished by heating it, its protective power is at the same time diminished, and Trumpp has confirmed this observation. Gengou, working on anthrax, found that no relation existed between agglutinative power and lysogenetic power, and Malvoz is of the same opinion; but Deutsch, in a recent communication, has succeeded in showing that in the single dose immunisation of guinea-pigs, agglutinins and protective bodies develop together; both appearing, both reaching their maximum, and both subsequently gradually diminishing in about the same number of days after the single intraperitoneal injection. The two processes, in fact, march side by side in clearly parallel, though not superposable, directions. We are therefore justified in strongly supporting a belief that a relation does, in fact, exist between the agglutinative power of a serum and its protective power, but that this relation is not one of proportion. And we may accordingly regard the relative agglutinating power of a given serum on different races of typhoid bacilli, or of different sera on the same race, as affording some sort of index of the relative protective power of the given serum against those races, or of the different sera against one and the same race.

NATURE OF THE RELATION BETWEEN AGGLUTINATION AND PROTECTION. AGGLUTININS.

If we now proceed to inquire in what way agglutination and protection are related, we are met at the outset by considerable difficulties. The nature of agglutination is unknown, and its mechanism is insufficiently determined.

Gruber believes that the effect of the action of agglutinins is to render the bacillary membrane viscous, so that the bacilli adhere on contact, though why they should come in contact at all in the case of non-motile or dead organisms is not stated. A somewhat similar view appears to be held by Nicolle, who also, confirming the observations first made by Kraus, has shown that the agglutinable substance is

present in quantity in the bacillary membrane, and diffuses out from it into the culture medium. Harrison has further recently found that it can be dissolved or digested out of the membrane by the action of the *pyocyaneus* ferment until the bacilli themselves become no longer agglutinable. Bordet, again, regards the process of agglutination as analogous to that of coagulation of blood; while Paltauf considers that the bacilli are mechanically entangled in the meshes of a coagulum produced in the ambient liquid; and Dineur maintains the formation of an adhesive substance on the bodies of the bacilli, and especially upon their cilia, so that they adhere, and the latter become entangled.

That the process is brought about by the action of certain substances formed in the animal body during the process of immunisation was almost universally believed until quite recently. Doubt was first thrown on this by the observations of Malvoz, who showed in 1897 that agglutination is not a special property of specific sera, but is producible also by certain comparatively simple chemical substances, as, for instance, formalin and sublimate. He has since found that safranin, vesuvium, fuchsin, acetic acid, lactic acid, and other substances have a similar action. More recently the same observer has discovered, at any rate so far as concerns anthrax, on which he worked, that similar substances are present in the cultures of the microbe concerned. He believes that the bacteria themselves carry with them products which in the presence of certain substances—bouillon, peptone solution, and the like—lead to agglutination of the microbes by the precipitation of colloidal substances by which they are entangled. He suggests, further, that in immunised animals the blood is merely charged with such colloidal substances derived from the culture media injected; and quotes, in support of this, his observation that after the injection of a considerable quantity of the premier *vaccin du charbon* into a dog, the blood is found to be already very highly agglutinative in twelve or fifteen hours, and thenceforward rapidly loses agglutinative power, since the agglutinating substance is excreted, as shown by Nicolle in rabbits, in the urine. He quotes also, in support of his contention, the statement of Gengou that the organs of the body, leucocytic exudations, and the white corpuscles of the blood, contain less agglutinating substances than the serum. But against this we may set the experience of Wyatt Johnston, and M'Taggart, drawn from their observations on their "dried blood method" of serum diagnosis, that whole blood contains considerably more agglutinating substance than the serum; as well as the discovery of Deutsch, that lung juice contains even more of the agglutinating substance than blood serum itself.

The facts recorded by Malvoz throw considerable light on the process of natural sedimentation, already discussed, and afford a new illustration of the fact that bacteria excrete substances which, as in the case of other excreta, are inimical to the activity and well-being of

the organism. But they do not by any means prove that the reaction which he obtains with formalin, or with culture fluids freed from bacteria by centrifugalisation, is identical with that produced by the serum of an immunised animal; nor do they show that agglutinating substances are not formed in the process of immunisation, but merely injected in the culture media. He himself says, speaking of the reaction which he obtains with the agglutinating substance found in a test tube culture of anthrax, and of the sedimentation observed in certain cultures of typhoid and other organisms, "Pseudo-reaction is the name given, and one has always differentiated this—I know not why—from true agglutination." The reason, of course, is clear, at any rate as regards typhoid, of which I can speak without hesitation, for the pseudo-reaction is in most cases microscopically distinguishable from the true agglutination, and certainly never approaches the appearance of a "complete" reaction. Moreover, if the agglutinating substance of the blood is entirely agglutinating substance injected during immunisation, it is impossible to conceive how, in the case of the horse, for example, the repeated injection of a certain number of cubic centimetres of culture into an animal possessing many litres of blood can give rise to a storage of this substance in the blood sufficient to produce complete agglutination by means of a serum dilution of, say, 1 to 1000. For even if the whole of the blood were replaced by culture medium, such a result would not be attained, since culture medium diluted to 1 to 1000 will certainly not give "complete" agglutination. And, further, if the agglutinating substance of immune blood were entirely a bacterial product, and not an immunisation product, it becomes impossible to comprehend the persistence of the agglutinative reaction in convalescent man for a year or more after an attack of typhoid fever, as noted by Wyatt Johnston and others, since the agglutinating substance of Malvoz is rapidly excreted in the urine. It is therefore quite impossible to accept the views of Malvoz as to the origin of the agglutinating substance of the blood.

We have already stated that the true agglutinative substances, or agglutinins as they are called, run parallel in their formation with the formation of the protective bodies during immunisation. But that they are not at all intimately connected with the latter is shown by the observations of Deutsch, that their production does not take place at the site of production of the protective bodies, in the lymphatic and spleno-medullary systems, but probably in the lung, in connection with the normal agglutinins of that tissue; and also by the fact noted by Pfeiffer, for cholera serum, that agglutinative power may have sunk to the level of that of normal serum, while protective power still remains increased.

We may, therefore, probably most suitably regard agglutination as an adjuvant, though not an essential, to the lysogenetic action of protective sera. Certainly the presence of small amounts of an agglutin-

ative serum considerably modifies the properties and growth of its bacillus *in vitro*; its presence in the living body probably assists, by the clumping and paralysis of the bacilli which it produces, the processes involved in bactericidal and lysogenetic action.

NATURE OF ANTITYPHOID SERA.

It is necessary to draw a clear distinction between antimicrobial sera, such as those of typhoid, and cholera and antitoxic sera, such as that of diphtheria. The latter have no bactericidal action. Antimicrobial sera possess bactericidal powers; do they also possess antitoxic powers? The question may probably be answered in the affirmative, at any rate for some cases, for Fränkel and Sobernheim have stated that cholera serum heated to 55° C. loses its bactericidal power, but still possesses protective power.

With regard to typhoid, the evidence is extremely conflicting. In the earlier work the phenomena following infection with typhoid bacilli were believed to be due to intoxication, and Brieger, Kitasato, and Wassermann held that the protective sera owed their properties to antitoxic constituents. Similar views were held by Beumer and Peiper and by Sirotinin. Sanarelli, however, found that his sera were antimicrobial and not antitoxic. Bitter, by treating rabbits with sterilised and concentrated typhoid cultures, obtained a serum which had an antitoxic power, and rendered innocuous an otherwise fatal dose of typhoid toxins. Pfeiffer, on the other hand, showed that the serum of convalescent man is antimicrobial, but without antitoxic power; while Stern stated that a similar serum was antitoxic; and Bruschettini prepared from rabbits one which was both antitoxic and antimicrobial. The horse serum prepared by Bokenham was found by him to be chiefly antimicrobial, and Pfeiffer and Kolle showed that their goat serum was entirely antimicrobial. These contending observations can only be harmonised, on the supposition that an antityphoid serum may be either antitoxic or antimicrobial, or both, being modified by the method of immunisation, age of cultures used, method of sterilisation, if only injected in a sterile condition, and by the various properties and virulence of the particular race of the bacillus employed.

Typhoid fever in man was also regarded as an intoxication due to the absorption of the products of bacilli which flourished in the intestine, and produced there certain characteristic lesions. The presence of typhoid bacilli in the mesenteric glands and spleen was then explained on a theory of "leakage" from the intestine. And certainly many of the features of the disease are markedly toxic. This view has, however, been strongly combated, and the observations of Wright and Semple among others go far to show that the disease is in reality a typhoid septicæmia. Indeed, if it were merely an intoxication, there

should have been no more difficulty in the preparation of a powerful "anti" serum, than in the case of diphtheria and tetanus. There is, in fact, a general invasion of the body by the bacillus, as is shown by its recognition, not only in the mesenteric glands and spleen, but also in other abdominal organs; in the kidneys, early and continuously in the urine, in the so-called rose-spots, and so on; and also in the metastatic abscesses which occur either during or following certain cases of the disease. We conclude, therefore, that the infection produces both toxic and septicæmic features, and that its protective or convalescent sera must possess both antimicrobial and antitoxic powers, though the latter appear to be considerably the less prominent. If such be the case, it becomes possible to explain the want of complete parallelism between agglutinative and protective power already indicated; for, as is abundantly demonstrated, no relation whatever exists between antitoxic power and agglutinating action. The agglutinative power is, therefore, in relation only with the antimicrobial power, and its parallelism with this may probably be complete. Indeed, admitting the hypothesis in question, we find a ready explanation of the objections which have been raised to Durham's original view, that agglutinative power and protection are related properties.

Thus, in the gradual loss of an acquired immunity, the antimicrobial power, and its related agglutinative power, may disappear before the much less highly specialised antitoxic power; the serum will therefore still possess some protective properties, although agglutination has been lost. But this was one of Pfeiffer's chief objections to Durham's theory.

SERUM THERAPY IN TYPHOID INFECTION.

Returning now to the question of protection by antityphoid sera, we have seen that various observers, and especially Pfeiffer and Kolle and Bokenham, have prepared sera having protective and curative value for guinea-pigs. The sera prepared in Berne have also been shown to possess protective power. With respect to their action in cases of typhoid fever in man, Professor Tavel has already published an account of four cases treated with the 1898 serum. In these cases the injections of the serum abolished the plateau stage of pyrexia, the fever falling by lysis, and the patients making rapid recovery. Further cases, treated with the 1899 serum, are about to be published, and I understand that the 1900 serum is at present undergoing extensive trial. The protective doses required by guinea-pigs are still large, even with the 1900 serum; but there is certainly more than sufficient favourable evidence to justify a very careful trial of this serum on man, especially as the inoculation method inaugurated by Wright and Semple does not appear to have proved completely protective.

While dealing with the question of treatment in man, mention

should perhaps be made of the so-called antityphoid extract of von Jex. This observer has employed the tissue serum extracted from the organs of rabbits which had been immunised against the typhoid bacillus, and claims to have obtained good results in the eighteen cases observed.

An element of consideration in certain cases of typhoid fever, which was emphasised especially by Vincent, is the importance of secondary and associated infections. Now we know that in this disease the virulence of the normal *Bacterium coli* becomes increased. This is also the case in dysentery, and for that disease an anti-dysenteric serum, prepared by Professor Belfanti in Milan, by the use of the *Bacterium coli*, has been found to give good results. It is open to question, therefore, whether in typhoid fever a similar serum may not, in association with antityphoid serum, give better results than antityphoid serum alone.

With regard, then, to the preparation of an antityphoid serum, which shall have real therapeutic value for man, we may note the following points:—

1. There is evidence that such a serum can be obtained by the immunisation of horses against the *B. typhosus*.
2. The initial stages of the preparation may be greatly shortened by adopting the method used by Bokenham.
3. A high degree of immunisation must be obtained, and the employment of living cultures in the later stages is desirable.
4. The serum must be made widely polyvalent by the use of as many and as largely different races of typhoid as is practicable.
5. The relative value of the serum obtained may be determined by a determination of its relative agglutinative power.
6. It is open to question whether the efficiency of the serum might not be further increased by immunisation of the horses against the *B. coli* also, or by the addition to the serum of a certain proportion of anti-coli serum from a horse treated with that organism.

THE THEORY OF EHRLICH.

We must now return to a brief consideration of some points in the theory of Ehrlich which bear upon the subject of the present thesis.

Enough has been said to show that for typhoid, at any rate, the differences of species, upon which Ehrlich has laid so much stress, do not prevent the preparation from the horse of a serum which shall have curative powers for other animals, and apparently for man. Whether this will prove to be true for other diseases also, is perhaps at present doubtful, since Sobernheim has shown that anthrax serum from the sheep has no protective action in rabbits; von Neisser obtained similar results. As concerns typhoid, the objections of Ehrlich are only supported by experimental evidence, when we come

to deal with animals treated with many times the M.L.D., a condition of great importance in the scientific investigation of the nature of immunity, but absolutely without parallel in human pathology, unless it be in the case of internal rupture of a large abscess cavity. And, even here, immunisation by absorption of bacterial products has previously had full play. In the process of immunisation certain changes take place in the blood and blood-forming tissues, which it is convenient to represent and materialise as a formation of certain "bodies" in the serum. But, in using the terminology of convenience, it is necessary to keep clearly in mind that the intricate physico-chemical processes concerned are in no way accurately represented by the comparatively simple reactions that occur between definite chemical substances brought together outside the living body. The tendency to express the processes with which we are as yet unacquainted in terms of the physical and chemical formulæ which we have already ascertained, but which are obviously inadequate to the conditions, has tended, probably more than anything else, to bolster up the last flickering hopes of the old vitalistic theories, and has given to neo-vitalism that standpoint which in reality belongs to intelligent materialism. Until we have gained an insight into the life-processes of protoplasm, and have laid bare those recondite physico-chemical changes and conditions which constitute life, and form the basis of normal physiology, it is impossible to formulate schemes of the abnormal events of cellular pathology. With this proviso, however, the terminology of Ehrlich is probably the best that could be employed.

Pfeiffer was the first to show that in bacteriolytic sera two "bodies" are present, a specific stable body (immune body) and an unstable body, present also in normal serum (complementary body), and that both are necessary for the occurrence of bacteriolysis. Fränkel and Sobernheim observed that cholera serum, heated to 55° C., loses its bactericidal power, and Bordet showed that if such serum have the serum of an unimmunised animal added to it, its bactericidal power returns. From this it followed that one of the bodies, readily destroyed by heat (the complement), exists also in normal serum. The researches of Belfanti showed further that the properties of artificially hæmolytic sera depend on the presence of similar bodies. Ehrlich and Morgenroth have greatly elaborated and extended these observations. They maintain that the complement is special to the species of animal concerned, and find that it possesses properties which render it comparable to a "ferment." And they hold that the specific nature of this body is of great importance, and explains the therapeutic failure of many of the sera which have been prepared. They claim, in fact, that an animal can only make use of its own complementary body in resisting the attack of an invading organism, but that this is, in the unimmunised animal, present only in limited amount; when, therefore, protective serum from another species of animal, whose complementary body

is useless except for its own species, is injected, there is found in the serum of the animal to be protected a deficiency of complement, and the immune body is therefore unable to protect, and lies useless. It is suggested, accordingly, that we require to seek for suitable means of supplying the needed complement. Wassermann, in a recent investigation, supports the same contention; he believes that, in order to increase the therapeutic action of a bactericidal serum, we must find a complement which is not only satisfactory to the immune body, but also does not lose its power of action when injected into the infected animal. Working with immune serum obtained from a dog, he found that the necessary complement was supplied for guinea-pigs by *fresh*¹ ox serum. But at the same time he showed thereby, perhaps unconsciously, that the complement of the guinea-pig is not so special but that it can be replaced by that of so considerably different an animal as the ox.

Ehrlich, Wassermann, and others have shown that if we find the equivalent of antiserum for the M.L.D., of a given organism for a given animal, *i.e.*, that amount of serum which protects against the M.L.D., and that if we now inject into the animal three times that amount of serum, with three times the M.L.D. the animal is not protected, but will die. And this is taken as evidence of deficiency of complement.

Let us examine the experiment a little more closely. Let d represent the M.L.D., then if the M.L.D. has been determined to within 10 per cent., say, $\frac{9}{10}$ of d was the nearest dose less than d found not fatal. That is to say, an immunised animal has natural immunity sufficient to account for a dose of $\frac{9}{10} d$. Therefore, in the case of the first injection, the antiserum equivalent was only actually engaged in protecting against $\frac{1}{10} d$. And, since the least amount of serum necessary to protect was taken, we may say that the dose of antiserum given was the protective equivalent of $\frac{1}{10} d$. If, now, we examine the conditions in the second case, we see that the serum injected would be protective against $\frac{3}{10} d$, and the organism itself is able to deal with $\frac{9}{10} d$. But the dose injected was $3 d$. There remains, therefore, a dose of $1\frac{8}{10} d$, against which no protective process whatever occurs, and obviously the animal dies.

If the M.L.D. had been determined to within nearer limits than 10 per cent., the result is more favourable to the argument; if within wider limits, only somewhat less favourable; but so long as the M.L.D. has been determined to within 50 per cent. even of its true value, the animal must die in this experiment, quite apart from any supposed deficiency of complement. And the greater the number of M.L.D.'s, and the number of serum equivalents given, the larger will be the dose of the bacillus for which no protection whatever is afforded, and the more rapidly will the animal die. And if in the preceding experiment

¹ The italics are mine.

the serum equivalent had been determined, not for the M.L.D. but for some multiple of this, as, for instance, twice, thrice, or five times the M.L.D., and the experiment continued as before, then we see, by a similar train of reasoning, that the serum given would similarly soon cease to be protective when the doses of infective material and serum were multiplied as before. Now Wassermann, confirming Pfeiffer, has also shown that, if we go above a certain degree of dosage of infective material, we may give as much immune serum as we will, yet the animal will die from bacterial multiplication. And, further, that we can give so much serum, even that the body fluids of the dead animal can protect another guinea-pig, so long as the degree of dosage referred to above is not approached. And Wassermann concludes that the first animal dies from lack of complement, and that its fluids are protective for the second, because they find in it the complement required.

But if now we consider the question broadly, and seek to determine what this "complement" may be, we shall first of all naturally inquire what are indicated as its characters and properties. And these we find are the following:—It is present in normal serum; it is present, however, in limited amount; it is replaceable sometimes by fresh serum from another species of animal (if we find the right species is Ehrlich's condition); it is destroyed by a temperature of 55° C.; it is of the nature of a "ferment" in action; it is not replaceable by dead stored serum; immunisation results in its production in quantity sufficient for protection. It must surely be impossible to consider these conditions for a moment without the conclusion becoming irresistible, that the mysterious complementary bodies in question cannot be other than the leucocytes, or rather those varieties of leucocytes (and their secretions), which, as Kanthack and Hardy showed on anthrax, are concerned in the destruction and digestion of bacteria. These are the bodies which are present in normal serum, but present in quite limited amount,—for the varieties concerned are not among the most numerous of the different kinds of leucocytes,—which are replaceable by fresh serum, in which they continue to live for some little time under favourable conditions, and which at any rate contains those leucocytic ferments or secretions which, broken down, have disappeared in older sera; which lose their activity after exposure to a temperature of 50° C.; which are not present,—nor their ferments,—in old dead sera; and which increase during the process of immunisation and become "educated," as Metchnikoff expresses it, to deal with the invading organism. Hence it is that dead immune serum has no bacteriolytic power on its bacillus, unless the experiment be made in the peritoneal cavity of an animal. And hence it is that Deutsch was able to show that the protective bodies are formed in the lymphatic and especially in the spleno-medullary system.

Here also we find the true explanation of the two experiments of Wassermann already quoted. In the first of these we have shown

that it was not the "complement" of the animal that was deficient, but the protective serum; and now we see that it is not that the "complement" of the protective serum cannot replace deficient "complement" in the animal to be protected, for the protective serum has no longer any "complement"—its leucocytes are dead and their products degenerated, but that it is in reality the immune body which is insufficiently supplied to the animal. And in the second experiment, where excessive doses of bacteria are injected, we have the following conclusions:—The protective leucocytes pre-existing in the blood are soon used up (if they be not actually repelled by negative chemiotaxis, attending the excessive dose of micro-organisms and their dissolved products), time is needed for the supply of further leucocytes by proliferation at their site of formation; meanwhile, the immune body is powerless to act alone, and the bacteria are continually multiplying. It becomes, then, practically a struggle for existence between the bacilli and the leucocytes, in which, with the dosage here given, the former win the day and the animal dies. And, obviously, the unused immune body in the fluids of the dead animal is capable of application in a second animal, whose leucocytes are submitted to no such great ordeal, but which have only a moderate dose of organism to deal with. It appears, therefore, on the view proposed, that the theory of the specialism of the "complement" for the species is unnecessary, and, if unnecessary, undesirable.

We are now also able to obtain a clearer view of the important differences separating antitoxic from antimicrobial sera. In the case of antitoxic sera, the formed antitoxine acts directly upon its toxine, and can so act *in vitro* as well as *in vivo*, but the immune body of antimicrobial sera, bactericidal to the bacilli, through the agency of its complementary leucocytes alone, acts only in conditions where those leucocytes can be supplied. And with regard to the question of agglutination, we can only now add (having previously concluded that it occurs in relation with antimicrobial power), that it is adjuvant to the leucocytes, and the associated immune body, by arresting the rapidly moving bacilli, and bringing them together into masses more easily approached and more conveniently attacked than are the free, single, active, tiny micro-organisms.

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