THE EFFECTS OF DOSAGE IN TYPHOID VACCINATION OF RABBITS.

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(With 7 Curves.)

It has long been known that the injection of very minute quantities of a suitable antigen is sufficient to induce a well-marked response in the animal into which they are introduced. Friedberger, for example (1902) demonstrated the appearance of antibodies in rabbits after the intravenous injection of $\frac{1}{2000}$th part of 1 milligram of cholera vaccine, and the data which have accumulated since the introduction of vaccine-therapy, as also the phenomena of serum-anaphylaxis in the guinea-pig, show clearly enough the remarkable sensitiveness of the animal body to at least certain classes of material directly introduced into its tissues. These minimal injections, however, do not produce the full antibody-response, of which the animal is capable. Friedberger in the paper quoted showed that a larger dose was followed by the appearance of a larger quantity of antibody, and the experiments of Ascher (1901) with typhoid vaccine indicate that, broadly speaking, the production of antibodies increases with the size of the dose. This is indeed generally recognised to be true when the doses in question are quite small (cf. Wright, 1909); but it was one of the objects of the work recorded in the present paper to ascertain whether in the case of typhoid vaccine it is true for any really considerable range of dosage. Even for small doses the necessity of injecting the different doses into different animals (Cole, 1904) has made the point somewhat troublesome to establish. Individual animals vary very greatly in their response to injections of the same size, whether single or repeated, and Stäubli (1904), working with typhoid vaccine in guinea-pigs, went so far as to deny that there is any relationship whatever

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between the quantity of bacteria introduced and the resulting formation of agglutinin. It is evident that conclusions can be based only on the average results of a considerable number of animals. Further it would seem to be evident that, if it exists at all, a direct relationship can hold good only within a definitely limited range of dosage. There must be a limit to the increase in production; and reactions of this kind, as estimated by the activity of the serum of the animals treated, tend even after repeated injections to approach a limit, beyond which they cannot be driven by any dosage. We have accordingly investigated this question afresh with typhoid vaccine and rabbits, using a wide range of doses and for each dose employing animals in numbers sufficient to allow of reasonable conclusions. The sera of these animals were tested in various ways detailed below.

The strain used for the preparation of the vaccine was one which had been isolated nine months previously from the urine of a typhoid carrier, who is known on definite bacteriological evidence to have been excreting the organism since an attack of enteric fever three years before. At the time of isolation, 0·1 c.c. of a 48 hours broth culture killed a guinea-pig in 24 hours. During the interval it had been kept in agar at room temperature, and its virulence appears to have altered a little between the time of first isolation and the date at which our experiments began; but that it remained fairly constant during the course of our work is shown by the following small table. The inoculations were made intraperitoneally.

Time	Dose	No. of guinea-pigs	Result
Beginning of experiments	2000 mills.	3	All died, 30 hrs.
	1000	3	Do. do.
	500	3	Do. do.
	100	3	2 died 30 hrs., 1 survived.
	50	3	2 survived, 1 died 12 days.
End of experiments	600	2	Both died, 30 hrs.
	300	2	Do. do.
	150	2	1 died 30 hrs., 1 survived.

This strain, which we shall call strain *S*, was the only one used in our experiments, with the exception of the tests for bactericidal action (see later). It was agglutinated microscopically by a powerful anti-typhoid serum in a dilution of 1:10,000, was actively motile, and gave the standard carbohydrate and other reactions. It was maintained by repeated subcultures in agar at room temperature, and this stock was never incubated at 37°. For use subcultures were made from a room temperature stock culture of 7—14 days old, and incubated

16—20 hours at 37°. The growth was then taken off in 0·85% NaCl solution, thoroughly mixed in a mechanical shaker, and the number of organisms contained in 1 c.c. of the suspension counted by a method which has an error of usually 2—5%, and at most 11%. The emulsion was then diluted with salt solution to the desired concentration. Throughout the whole of this work the suspensions were counted in this manner before use; and we consider that the increased accuracy obtained by working with suspensions of standard strength justifies the not very great additional labour involved.

The vaccine, which was freshly made for each series of experiments, was prepared by heating a counted emulsion at 60° for half an hour, and then diluting so that the required dose was contained in 1·5 c.c. This amount was then injected subcutaneously over the abdomen of each of ten rabbits of approximately equal weight, from each of which a sample of blood had been previously withdrawn. At intervals after the inoculation blood was taken from the marginal ear-vein of each of the ten rabbits, the serum taken off after 24 hours, and equal parts of the ten sera mixed together to form one sample. This was done on successive days after the inoculation, as indicated in the tables, and a series of samples obtained, each of which represents the whole of the ten rabbits taken together.

By this method of pooling the sera we hoped largely to obliterate the differences in the individual sera, which are particularly marked in the case of rabbits and typhoid bacilli, and to obtain the representative average required. A further and important advantage of the method lies in the fact that only a small quantity of blood need be taken from any one animal at each bleeding. In order to carry out the examination of the sera in the manner we proposed, about 5 c.c. of serum was necessary. This represents approximately 10 c.c. of blood, and the withdrawal of so large a quantity every day or every other day for a fortnight from an animal of the size of a rabbit would seriously affect the production of antibodies. Using ten animals we required only 1 c.c. of blood from each, and it was hoped thereby to minimise the effect of the repeated blood-letting.

In the earlier part of the work, each sample was tested for its content of various antibodies on the same day as we obtained the sera of which it was composed; and this method was adhered to throughout in the testing of the opsonic power of the whole serum, when it was essential that the serum should be fresh. But in other cases where heated serum was used, we subsequently adopted the more convenient method of

heating the samples for 30 minutes at 56°, and then keeping them in the cold and testing all or a number of them together on the same day. We thus examined a number of samples for, say, bactericidal action and agglutination on one day, and on the next day for, say, precipitating and tropic action, and so on. The error due to the different ages of the samples we did not find to be significant within the limits of time used, if they were reheated at 56° on the day of testing.

Five groups of rabbits were examined, each consisting of ten animals. To the first group were given 20 million bacilli per animal, to the second 100 million, to the third 500 million, to the fourth 2500 million, and to the fifth 12,500 million. The dose of typhoid vaccine usually given as a first inoculation to an adult man is 500 million, or from 7—8 million per kilo., taking the average weight of a man as about 70 kilos. The average weight of the rabbits used was 2½ kilos., so that the first group received 8 million per kilo. or approximately the same dose, weight for weight, as is given to a man, and the fifth group received over 600 times as much. The doses were in nearly all cases well borne by the animals. Only two out of the 50 died within 14 days of the injection, both being in the first group with the smallest inoculation. There was a considerable loss of weight after the injections, but this did not increase proportionately to the size of the dose—*e.g.* a week after the injection, the average loss of weight in the animals of the first group was 10%, in those of the fourth group 5%, in those of the fifth group 8%. It is perhaps worth noticing that the greatest loss of weight and the only deaths occurred in the group with the smallest dose; but this cannot be attributed to a progressive change in the strain, since this group was in fact treated third in order of time after two of the other series had been completed.

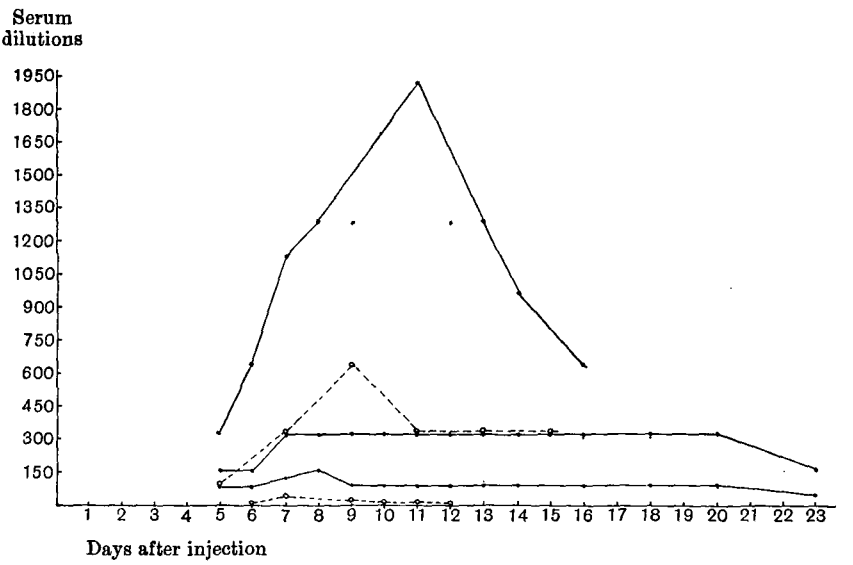
The samples of serum were examined for (1) their power to agglutinate the homologous bacillus; (2) their capacity to give a precipitate with an extract prepared from this bacillus; (3) their capacity to deviate complement, when conjoined with such an extract; (4) their bactericidal action; and (5) their capacity to promote phagocytosis. The results are tabulated under these five headings.

1. *Agglutination.*

This was observed macroscopically. To 1 c.c. of successive dilutions of the sample under examination was added 1 c.c. of a suspension of strain *S* containing 1500 million organisms per c.c. After thorough mixing the tubes were incubated two hours at 37°, when the results

were read; and after standing overnight at room temperature (about a further 18 hours) a second reading was taken. The latter was the reading adopted, but the earlier reading was sometimes of value in enabling one to decide definitely on slight differences between two samples. The standard chosen was the lowest dilution in which definite agglutination was visible.

In Table I is given an example of the complete examination of one group, and in Table II the results obtained with all the groups.



Curve 1. Agglutination. From above downwards the curves represent the series after 12,500, 2500, 500, 100 and 20 millions.

In the first two groups examined the serum was tested both fresh and after heating, but as identical results were obtained, the later series were tested with heated serum only. The results are perhaps better seen when shown graphically as in Curve 1. In most cases the dilution of the serum in the successive tubes of a test fell by one-half, so that a reading of, say, 1 : 40 means that the value of the serum lay between 1 : 40 and 1 : 80. The curves therefore cannot be smooth, and indicate with only approximate accuracy the change in agglutinin-content from day to day. They are of the familiar type, and, like the other curves representing our results, show the usual features of curves of antibody-production.

TABLE I.

Agglutination—Dose = 100 *millions.*

Serum-dilution	Before inoculation	Days after inoculation: 5	6	7	8	9	10	11	12	13	14	16	18	20	22
1 : 5	0	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
1 : 10	0	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
1 : 20	0	+++	+++	+++	+++	+++	+++	+++	++	++	++	+++	+++	+++	+++
1 : 40	0	++	++	+++	+++	++	++	++	++	++	++	+	++	+	++
1 : 80	0	++	++	++	++	+	+	+	+	+	+	+	+	+	0
1 : 160	0	0	0	±	+	0	0	0	0	0	0	0	0	0	0
1 : 320	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1 : 640	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

+++ = complete, ++ = marked, + = partial, 0 = no agglutination. Control without serum : 0.

TABLE II.

Agglutination: all series.

The numbers indicate the lowest active dilution of serum.

Dose of vaccine	Before injection	Days after inoculation: 3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	23
20×10^6	<5	<5	<5	<5	5	40	—	20	10	10	5	5	<5	—	—	—	—	—	—	—
100×10^6	<5	—	—	80	80	120	160	80	80	80	80	80	80	—	80	—	80	—	80	40
500×10^6	<5	—	—	160	160	320	320	320	320	320	320	320	320	—	320	—	320	—	320	160
2500×10^6	<5	—	—	80	—	320	—	640	—	320	—	320	—	320	—	—	—	—	—	—
$12{,}500 \times 10^6$	<5	—	—	320	640	1120	1280	1280	—	1920	1280	1280	960	—	640	—	—	—	—	—

The conclusions which may be drawn from these results will be discussed later, but we may note that, as the dose of vaccine is increased, the maximum agglutinating power of the serum also increases, while the date at which this maximum is reached gets later.

2. *Precipitation.*

An extract was prepared from strain *S* as follows. Several Roux bottles of agar were inoculated, each with 1 c.c. of an 18 hours broth culture and incubated 48 hours at 37°. To each bottle was then added 20 c.c. of distilled water, into which the growth was swept off and the whole mixed. After heating one hour at 60° and allowing to cool, carbolic acid to 0·5 % was added, and the suspension then frozen hard. It was slowly thawed at room temperature and shaken over night in a mechanical shaker at 37°. It was again frozen, thawed and shaken, and finally centrifugalised. The supernatant fluid was distinctly opalescent but microscopically free from bacilli and was sterile. This stock was kept in the cold room, and one in ten dilution in 0·85 % NaCl solution used for testing the samples of serum. To 1 c.c. of successive dilutions of the serum was added 1 c.c. of the extract-dilution, the whole mixed, and placed at 37° for four hours. A preliminary reading was then taken and the tubes examined again after standing all night at room temperature for final reading.

As in the case of agglutination the standard selected was the lowest dilution of the serum in which a definite precipitate was visible to the naked eye. This was usually easy to determine. The precipitum had the form of a slight loose delicate coagulum, of the appearance of a tuft of fine wool, never very copious but quite definite in suitable light and characteristic, floating up readily on gently shaking the tube. The results obtained are grouped together in Table III. (An example of a whole series is not given, as we obtained no irregularities or zone phenomena.)

TABLE III.

"Precipitation."

Dose, millions	Before injection	Days after injection															
		5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
20	0	0	0	20	—	10	10	10	10	10	5	—	—	—	—	—	—
100	0	10	40	160	160	80	40	20	20	20	10	—	10	—	10	—	10
500	0	40	320	320	320	320	240	240	240	160	160	—	80	—	80	—	80
2500	0	10	—	40	—	80	—	80	—	40	—	40	—	20	—	—	—
12500	0	160	160	160	1280	640	—	640	640	640	640	—	640	—	—	—	—

Here again we notice a tendency for the maximal response to be reached at a later date with the larger inoculations, up to the dose of 2500 millions. With the highest dose, however, the maximum has regressed. Further the height of the maximum does not increase regularly with the increase in dosage. The injection of 2500 millions is followed by a serum-value of only 1 in 80, instead of the 1 in 600 or 700 one might expect. In any consideration of the significance of such variations in the regularity of response, it is necessary to remember that in the case of precipitation as of complement-fixation the results obtained are subject to an error which does not equally affect the other reactions examined. This lies in the difficulty of obtaining a constant antigen. A bacterial extract, kept in the cold and dark, undergoes a gradual alteration of the particles it contains, as is shown by the deposit which falls out as time goes on, and this change in the state of the particles affects the result of the interaction of the serum with the extract. When an experiment lasts over several weeks, as did those described, it is impossible to use the same extract throughout and still obtain results that are really comparable. It is necessary to make fresh extracts from time to time. But this proceeding introduces a fresh error, for no two extracts, though prepared in apparently quite the same way, are exactly alike in their reactions with the same serum. In our series the first and fourth were tested with the same extract, the second and third with a second extract, and the fifth with a third, all these extracts being prepared in the same way but at different times. Not much stress, therefore, can be laid upon the actual figures obtained, as indicating the relative strengths of the sera in question.

But however inexactly these figures represent a relationship between the dose injected and the consequent production of "precipitin," they are at least approximately accurate for any one series and the extract with which that series was tested, and in this respect they are somewhat remarkable. It is the current view, at least in this country (Chapman and Welsh, and cf. Dean, 1911, who gives a number of references), that in precipitin reactions the precipitum formed, the actual material which falls out of solution, is derived mainly from the serum of the treated animal and only to a small extent, if at all, from the antigen. In accordance with this it is the general experience that, while the antigen may be diluted to an enormous extent before the reaction ceases to form a visible precipitate, dilution of the anti-serum is practicable only within a comparatively small range, even with high-grade sera. Thus it is a good serum which reacts in a dilution of 1:100, and sera

which react in dilutions of more than 1:300 are quite exceptional. But in our experiments, when the sera would ordinarily be considered as of low grade, we obtained precipitation in dilutions of 1:160, 1:320 and even 1:1280.

Again, while a serum of so high a value as 1:1000 would in the ordinary way permit of a dilution of the antigen to exceedingly high figures (*1:100,000* or figures of that order), the reaction, which we obtained, ceased to appear when the antigen was diluted to 1 in 80. The whole of the samples of our last series were tested with extract-dilutions of 1:60 and 1:80, as well as 1:10, and no reaction was obtained with any sample in the 1:80 series, and only in a few tubes of the 1:60 series. Further, the nature of the coagulum, which has already been described, differs both from the copious flocculent precipitate and the general opalescence or turbidity obtained in strong and feeble reactions of the ordinary type. (Such precipitate we never obtained with any sample in a dilution of 1 in 2.)

These facts suggest that we are dealing here with a phenomenon which is quite distinct from the precipitation commonly described. They suggest that possibly the precipitate or deposit which we obtained is really an agglutination-phenomenon, an agglutination, however, not of bacilli but of the shells or particles of bacilli contained in the extract, and is not a precipitum in the sense in which that term is ordinarily used in connection with precipitinogen-precipitin reactions. This question requires a separate investigation, which is at present being undertaken.

3. *Complement-Fixation.*

We obtained results here with only one series, viz. after the injection of 500 millions. Two other series were also tested, those of the 20 million and 100 million injections, but no fixation whatever was obtained with any of the samples in these series by the technique adopted. Since we should have had only three determinations, even if (as was probable) the two largest doses had been followed by appreciable responses, and these would in any case have been subject to the limitations involved in the use of extracts, we did not examine the last two series, and include the results of the 500 million series only as an example of the kind of curve to be obtained. It is, however, not without interest that the small doses did not give us any reaction.

The technique adopted was essentially that in common use. To 1 c.c. of a 1 in 40 dilution of extract (prepared as described above)

was added 1 c.c. of successive dilution of serum, and 0·5 c.c. of fresh guinea-pig serum diluted 1 in 10. After 1 hour at 37°, 0·5 c.c. of a 5 % suspension of washed ox blood-corpuscles was added, with 0·5 c.c. of a dilution of haemolytic amboceptor standardised with the same complement to contain 3 lytic doses. The mixture was then incubated two hours at 37° and, a preliminary reading having been taken, was left overnight in the cold, and then the final examination made. The haemolytic amboceptor was derived from rabbits immunised to washed ox-corpuscles, and the standard taken was the lowest dilution of the sample, with which definite inhibition of haemolysis occurred. The usual controls were always made.

TABLE IV.

Dose of vaccine	Before injection	Days after injection												
		5	6	7	8	9	10	11	12	13	14	15	17	19
20×10^6	0	No reaction on any day.												
100×10^6	0													
500×10^6	0	10	20	20	20	20	40	40	20	20	20	10	–	0

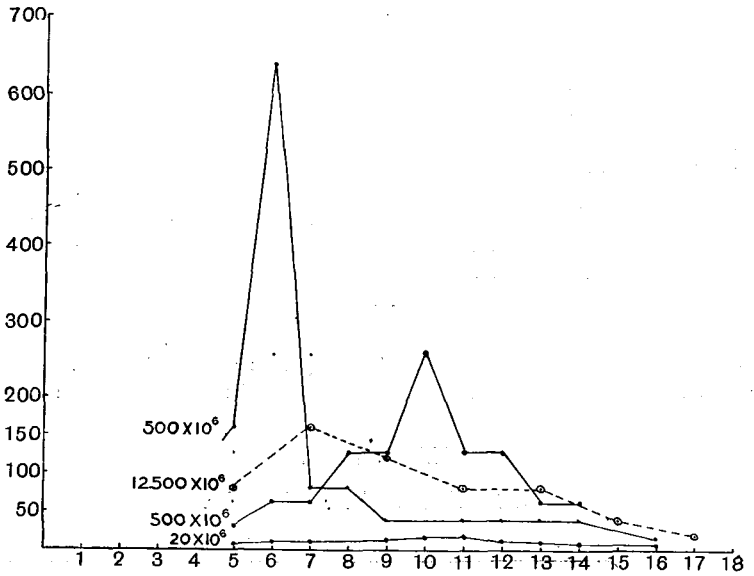
4. *Bactericidal Action.*

In testing this power of the serum we determined the smallest quantity of each heated sample, which in conjunction with a fixed quantity of fresh normal serum as complement killed all or all but one of a definite number of typhoid bacilli in a given time. Of each sample a series of dilutions in 0·5 c.c. volume was prepared, these dilutions being made in broth. To every tube was added 0·5 c.c. of a 1 in 2 dilution in salt solution of fresh normal rabbit-serum; and finally 3000 typhoid bacilli were added, contained in 0·5 c.c. of broth. The total volume thus amounted to 1·5 c.c. The necessary controls, which will be found detailed in Table V, were invariably prepared. We used as test-organism another laboratory strain of typhoid, and not strain *S*, which is rather resistant.

Immediately after mixing, two agar plates were made from the tube which contained only bacilli and the fresh normal serum, and also two plates from some one other tube. After incubating for 4 hours at 37°, two plates were made from every tube; the tubes were then filled up with broth and incubated overnight at 37°. In plating 5 drops were delivered into each plate from dropping pipettes standardised to deliver 0·02 c.c. per drop, only two pipettes being used for the whole

series (sterilised between each using). The plates were incubated at 37°, and the colonies counted, the mean of the numbers on the two plates being taken.

This technique differs from that usually employed in two points, the small number of organisms used and the large quantity of broth. The 1·5 c.c. of the mixture contained 3000 bacilli at the outset, so that on plating 5 drops or 0·1 c.c. 200 organisms were added to a plate. Of these 200 only 60–70% grew on agar at 37°, and the plates made at once after mixing show the easily counted number of 120–140 colonies. In a series of experiments carried out over several weeks,



Curve 2. Bactericidal action. (The scale for the 12,500 and 2500 million series is reduced one-fourth.)

in which the suspension of organisms was made sometimes by one of us, sometimes by the other, the actual counts nearly always lay between 100 and 140 in plates made at the beginning of the experiment, and the mean count in all experiments was 125. With this small number of organisms we found it impossible to use the small quantities of broth (*e.g.* 2 or 3 drops in a total of several c.c. of salt solution) which other workers have found sufficient. The bacilli died out steadily in the salt solution alone without the addition of any serum, and it was only when a considerable quantity of broth was present that their

numbers remained undiminished. Whether this is to be attributed only to an increased rate of multiplication induced by the additional broth or to an actual diminution in the death-rate was not determined.

In Table V are given the results of the examination of one series, and in Table VI a summary of all the series taken together. The curves are shown in Curve 2.

TABLE V.

Bactericidal action; injection = 2500 × 10^6 *bacilli.*

Serum dilutions	Before injection	Days after injection					
		5	7	9	11	13	15
1 : 5	many	0	0	0	0	0	0
1 : 10	many	0	0	0	0	0	0
1 : 20	many	0	0	0	0	0	0
1 : 40	many	0	0	0	0	0	0·5
1 : 80	—	0·5	0	0	0	0	10
1 : 160	—	4	0	3·5	36	86	171
1 : 320	—	60	20	30	271	many	many
1 : 640	—	277	178	175	many	many	many
1 : 1280	—	—	many	many	many	many	many
1 : 2560	—	—	many	many	many	many	many

Fresh Normal Serum + Broth: after mixing, 128.
after 4 hours, many (about 320).
Fresh Normal Serum + Sample before injection (1 : 5): after mixing, 135.
0·5 c.c. of all samples 1 : 5, and of fresh normal serum 1 : 2, in 5 c.c. broth: all sterile.

TABLE VI.

Bactericidal action: all series.

Dose of Vaccine	Before injection	Days after injection													
		5	6	7	8	9	10	11	12	13	14	15	16	17	18
20 × 10^6	not in 1	8	10	10	—	12	16	16	12	10	8	—	8	—	—
100 × 10^6	<2	64	128	256	128	128	256	128	128	128	64	—	64	—<16	
500 × 10^6	<2	128	256	256	512	512	1024	512	512	256	256	—	—	—	—
2500 × 10^6	<5	80	—	160	—	120	—	80	—	80	—	40	—	20	—
12,500 × 10^6	<10	640	2560	320	320	160	—	160	160	160	160	—	80	—	—

From these figures it appears that here again we have an increasing maximum with an increase of dose. The fourth series, when the dose is 2500 millions, is distinctly out of sequence, and this point will be referred to subsequently, but the other series show a rise from 1 in 16 with the smallest dose to 1 in 2560 with the largest dose. As in the case of the precipitation-values, however, the actual figures can be taken only as indicating approximately the relative strengths of the

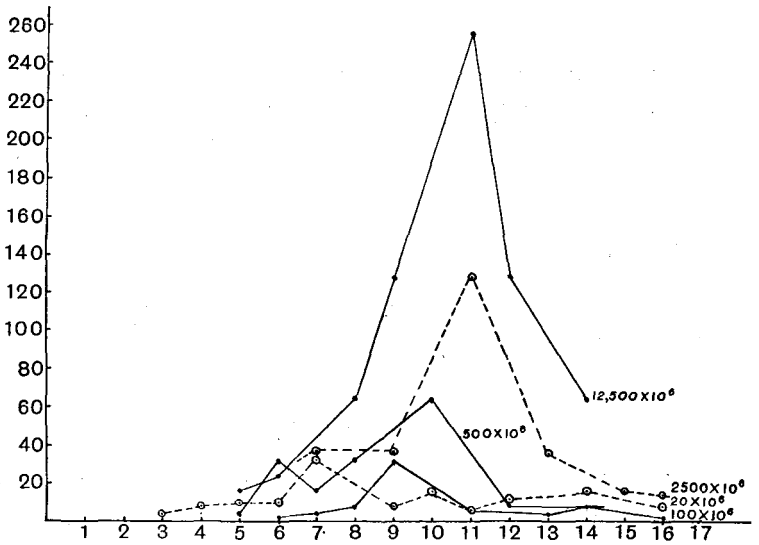
different series. These were necessarily tested with complement derived from different animals, and rabbit sera vary very markedly in their bactericidal power. Even when the pooled serum of two or three animals is used as complement (which was our usual practice), the difference in complementing power between two batches is still very marked, and a sample of immune serum will give a much higher value with one than with another. This is apparent in the controls of the normal serum alone. With the technique adopted, 0·5 c.c. of a 1 in 2 dilution of normal serum had considerable effect on the number of living bacteria found at the end of four hours, as compared with the numbers in a tube containing no serum at all. In most cases the numbers had only doubled or trebled themselves. But not infrequently the numbers actually fell, and on rare occasions all the organisms were killed. Experiments, in which diminution of this kind occurred, were rejected, and we accepted only those in which the bacteria in the complement-control tube had increased to two or three times their original number. But this variability of the power of the complementing serum makes it impossible to look upon the figures obtained as giving more than a general indication of the relative strengths of the maxima in different series.

5. *Phagocytosis.*

In testing the capacity of the samples to promote phagocytosis the dilution-method was employed (Klien, 1907). Successive dilutions of the sample were made and these dilutions were mixed with bacteria and leucocytes for 10 minutes at 37°. Smears of the mixture were then made, stained with Giemsa's solution and the average number of organisms taken up per leucocyte ascertained by counting in the usual way. From 100–300 leucocytes were counted of each mixture examined, the average number per slide being 120 in 80,000 cells. A control was prepared at the same time in which normal serum diluted 1 in 2 with salt solution replaced the sample-dilution; and the lowest dilution of the sample which gave a higher count than the control was taken as the value of that sample for our purpose. Human leucocytes were used, prepared in the way customary in the opsonic technique, and a bacterial suspension of 2000 millions in 1 c.c. (strain *S*).

Three methods were adopted in testing the activity of the samples. In the first the serum was used fresh after 24 hours on the clot, and

equal volumes of serum-dilution, bacteria, leucocytes and salt solution were mixed together, the control containing fresh normal serum. In the second, heated serum replaced the fresh serum of the first method, the control containing heated normal serum. In the third, heated serum was again used but complement was supplied by replacing the salt solution volume by one volume of fresh normal rabbit serum, the control containing heated normal serum instead of the immune-serum of the sample. The fresh normal serum used in the controls in the



Curve 3. Fresh serum phagocytosis. (The 12,500 and 2500 million curves are reduced one-half.)

first and third methods was derived from the same rabbit throughout, and the heated normal serum of the controls was the pooled serum of the rabbits of the same series before injection with the vaccine. As may be seen from Table VIII subjoined, normal serum induced remarkably little phagocytosis of strain *S*, indeed not much more than what occurred in salt solution alone, and the presence of complement made little appreciable difference.

As illustrations of the results obtained in this way we reproduce in Table VII the data of the examination of one series with fresh serum; and in Table VIII the figures obtained by all three methods with one sample. In Table IX are summarised the results of the examination of all the sera, the figures in the columns under each day indicating

the lowest dilution which gave more phagocytosis than the corresponding control. Cf. Curve 3.

It is of course a familiar observation that short exposure to moderate heat greatly reduces the opsonising power of an immune serum, without in general completely destroying it. This phenomenon is brought out in these figures got by the dilution method at least as clearly as by the usual method of comparing the phagocytic indices of undiluted serum in the fresh and the inactivated states. They further bring out, and even more clearly than is possible by the usual methods, the very marked influence of the addition of fresh normal serum or complement. This is best shown by an example, and we take the figures from the 500 million series on the 10th day after injection. Here the

TABLE VII.

Phagocytosis with Fresh Serum; Injection, 500 *millions.*

Serum dilution	Days after injection						
	5	6	7	8	10	12	14
1/1	5·26	—	—	—	—	—	—
1/2	2·68	14·41	7·63	—	—	2·74	—
1/4	1·27	—	5·60	11·28	—	2·55	2·70
1/8	0·72	3·20	—	—	11·84	0·91	1·64
1/16	0·32	1·52	1·47	1·83	—	0·51	0·31
1/32	—	1·29	0·63	1·08	2·66	0·42	—
1/64	0·35	0·88	—	0·67	1·51	—	0·13
1/128	—	—	—	—	0·56	—	—

Control of Normal Serum 1/2 taken as 1·00.

TABLE VIII.

Phagocytosis: 12th day after injection of 100 *millions.*

Serum dilutions	Fresh serum	Heated serum	Heated serum + Complement
1/1	6·73	0·64	—
1/2	2·65	0·49	—
1/4	1·15	0·25	—
1/8	0·26	0·23	—
1/16	—	0·10	2·84
1/32	—	—	1·34
1/64	—	—	0·84
1/128	—	—	0·42
1/256	—	—	0·35
1/512	—	—	0·20
Control	0·37	0·31	0·30

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TABLE IX.

Phagocytosis by the three methods with all doses. The numbers indicate the lowest serum-dilution found.

Dose	Method	Days after injection															
		3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
20 millions	Fresh	4	8	10	10	32	—	8	16	9	12	—	16	—	8	—	—
	Heated	—	No reaction obtained on any day.														
	Complemented	—	256	—	512	512	—	256	256	128	256	32	16	—	2	—	—
100 millions	Fresh	—	—	1	2	4	8	32	—	6	6	4	8	—	2	—	—
	Heated	—	—	2	—	4	4	8	8	2	2	2	—	—	2	—	—
	Complemented	—	—	32	128	128	512	1024	512	—	256	128	256	—	—	—	32
500 millions	Fresh	—	—	4	32	16	32	—	64	—	8	—	8	—	—	—	—
	Heated	—	—	16	16	—	16	—	32	—	32	—	16	—	—	—	—
	Complemented	—	—	128	2048	1024	1024	1024	2048	1024	—	—	256	—	—	—	—
2500 millions	Fresh	—	—	—	—	64	—	64	—	256	—	64	—	32	—	28	—
	Heated	—	—	—	—	16	—	64	—	64	—	32	—	16	—	—	—
	Complemented	—	—	—	—	512	—	1024	—	4096	—	512	—	256	—	64	—
12,500 millions	Fresh	—	—	32	48	—	128	256	—	512	256	—	128	—	—	—	—

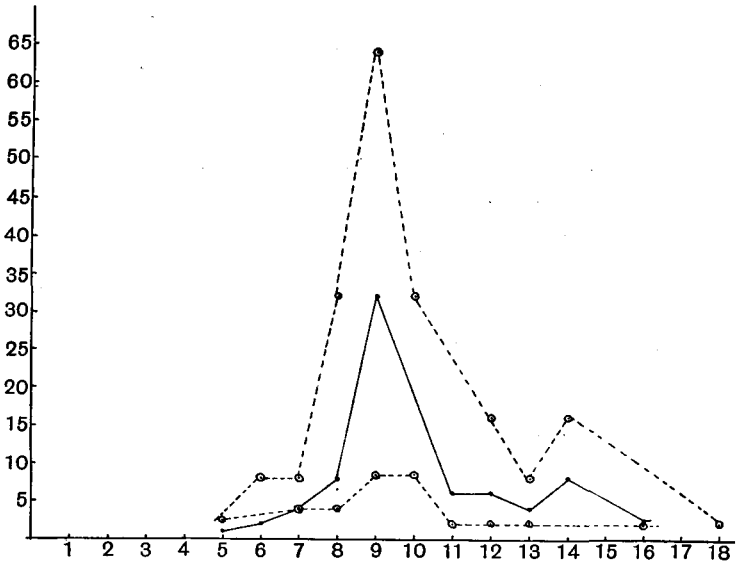
heated sample ceased to be more active than heated normal serum, when it was diluted beyond 1 in 32. On the addition of fresh serum, however, the heated serum of the sample proved much the more active of the two, and it was not until the sample had been further diluted down to 1 in 2000 (*i.e.* 64 times more) that its action again equalled that of the normal heated serum. There is here no question of addition or summation of the effect of the heated serum acting alone upon the effect of the fresh serum also acting alone, since the actions of the two heated sera, the normal and immune, are equal at 1 in 32, and the fresh serum should affect both equally if it were a mere summation effect. There can be little doubt that the power of the heated immune serum is greatly and (as one knows on other grounds) specifically reinforced by the addition of the fresh normal serum or complement. The sensitisation of the bacteria, then, in the experiments where fresh serum is added, is a process of the complement-amboceptor type[1], and involves at least two factors, one thermolabile contained in the fresh serum and the other thermostable in the heated serum.

We should expect then the reaction of the fresh unheated immune serum to be also of this type, and our figures supply a further confirmation of this view, which is that of G. Dean (1907), Cowie and Chapin (1907), and other workers. In other complement-amboceptor reactions, it is known that it is the so-called amboceptor which rises and falls during the process of immunisation, the complement remaining relatively little affected. If that is true here, the variations in phagocytic power of the serum from day to day are due to variations in the thermostable factor, and in our two methods of testing with whole fresh serum and heated serum to which complement is added, we are really measuring the same thing, viz. this thermostable factor. The curves showing the variation should therefore be similar for the two methods and follow the same course, rising and falling together throughout. We can hardly expect them to be exactly alike, since, apart from the considerable experimental error, dilution by the one method reduces both the active factors, by the other only the thermostable factor, but there should be a general parallelism of rise and fall. This is what in fact we do find. In Curve 4 the figures for one series are plotted in the same chart, and it will be seen that the similarity between the upper two curves is very marked. The other series show the same general agreement

[1] We use these terms only as the names currently employed to describe phenomena of a certain class; and not as terms which in any way represent the mechanism of the reactions concerned.

TABLE X.

Dose	Agglutination	Precipitation	Bactericidal action	Phagocytosis: Fresh serum	Phagocytosis: Complemented	Phagocytosis: Heated serum
20×10^6	40	20	16	32	512	—
	7th day	7th day	10–11th day	7th day	6–7th day	—
100×10^6	160	160	256	32	1024	8
	8th day	7–8th day	7 & 10th day	9th day	9th day	9–10th day
500×10^6	320	320	1024	64	2048	32
	7th day *et seq.*	7–9th day	10th day	10th day	6 & 10th day	10–12th day
2500×10^6	640	80	160	256	4096	64
	9th day	9–11th day	7th day	11th day	11th day	9–11th day
$12{,}500 \times 10^6$	1920	1280	2560	512	—	—
	11th day	8th day	6th day	11th day	—	—



Curve 4. Phagocytosis: by all three methods. The curves from below upwards at their highest points are in order (1) heated serum; (2) fresh serum; (3) complemented serum (reduced to one-sixteenth). Dose: 100 millions.

between the curves of the fresh serum and the complemented serum; and we believe that they represent the same phenomenon measured in different ways, viz. the variation in the thermostable factor.

In the curves of this kind where the results of all three methods of testing are plotted on one chart, it is noticeable that while the two

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upper curves follow the same course fairly closely, the third curve, that of the heated serum alone, does not show nearly so good an agreement. Its maximum does not coincide exactly with theirs in time, and it does not follow their irregularities very closely. The figures are perhaps not very good (the actual counts of the bacteria in the leucocytes are always low and therefore subject to a considerable error), and the differences are not so striking that we should care to lay very much stress on them. If however they are regarded as accurately representing the state of the sample, they have very considerable significance. We have seen that the capacity of an immune-serum to promote phagocytosis is mainly due to a complex action of the complement-amboceptor type. But when this is admitted, it is no longer necessary to suppose that the phagocytic power of heated serum is due to the amboceptor of the complement action. It may equally conceivably be due to the action of quite a different substance acting alone and always independently of complement, although its presence is masked by the more powerful complement-amboceptor action, if the latter is available. This is the view of Neufeld, who gave the name of bacteriotropin to this independent thermostable body, and it agrees with the common experience in other reactions that amboceptor is typically ineffective by itself. On this view there would be no reason to expect an agreement between the curve of heated serum and the other two curves, and the lack of coincidence which we actually find is at least suggestive. The differences we have met with, however, are not sufficiently striking to permit of any very definite conclusions from these experiments.

The results of the separate investigations of these various antibody-reactions are brought together in abbreviated form in the last table, No. X. In this the maximum value of the pooled serum of the 10 rabbits is tabulated opposite the dose of vaccine to which it corresponds, and then is also tabulated the date after injection on which that maximum value was obtained. From these figures some conclusions may be drawn, which for the sake of clearness are grouped together under three headings, (1) the date of the maximum response, (2) the relation of the response to the dose, (3) the relation of the antibodies to one another.

1. *The date of the maximum response.* It is apparent that in the agglutination, phagocytosis and "precipitation" series, the maximum value of the serum after injection is reached at a date which gets progressively later, as the dose is increased. The date for the last precipitin value has regressed but this is the only exception in the

series. A similar postponement of the apex of the opsonic curve has been already described by Wright as occurring in man after too large a dose of bacterial vaccine, but so far as we are aware has not hitherto been noticed in connection with agglutination. Wright connects this delay with the occurrence of a negative phase which follows shortly after the injection. During this phase the serum-value is depressed below that which it possessed before the injection was given and this condition persists for a time, which varies, generally speaking, according to the magnitude of the overdose. When this period is over the serum-value rises in the typical case beyond its original value and reaches a maximum in the usual way; but this interval of depression naturally makes itself felt by a delay in the arrival of the maximal value. The phenomenon is most easily demonstrated in individuals, whose serum already possesses a fairly high value and was shown by Salomonsen and Madsen, so long ago as 1897, to occur in antitoxin horses after the injection of diphtheria toxin. In our animals we did not examine the serum before the 3rd or in most cases the 5th day after injection, by which time the negative phase, if present, might well have passed off, and it would in any case have been rather difficult to demonstrate in animals previously normal with serum-values so low as those we found before injection. The curves, however, do not suggest that the four days' delay in reaching the maximum value with the largest dose is due simply to a negative phase of corresponding duration. If that were so, we should expect the curves with late maxima to cross the base line later than the curves with early maxima, and therefore to intersect these early curves during the rise to the apex: and this does not appear to be the case, *vide* the curves of fresh serum phagocytosis, no. 3, and agglutination, no. 1. We incline rather to attribute the delay to a longer period of absorption of the large dose from the site of injection. The small dose is absorbed comparatively rapidly from the subcutaneous tissues; whereas with a large dose this process takes a longer time. There is therefore a more prolonged stimulation of the cells in the latter case, with a correspondingly prolonged period of rise in the curve. This view agrees better with the fact that the curves do not intersect, but as a general rule the curve with the higher maximum is higher throughout on each of the observed days than the curve with low maximum—at any rate during the period of rise. The phenomenon recalls the delay in the development of the anaphylactic state after a large dose of sensitising antigen.

No such delay occurs with the bactericidal action. On the contrary

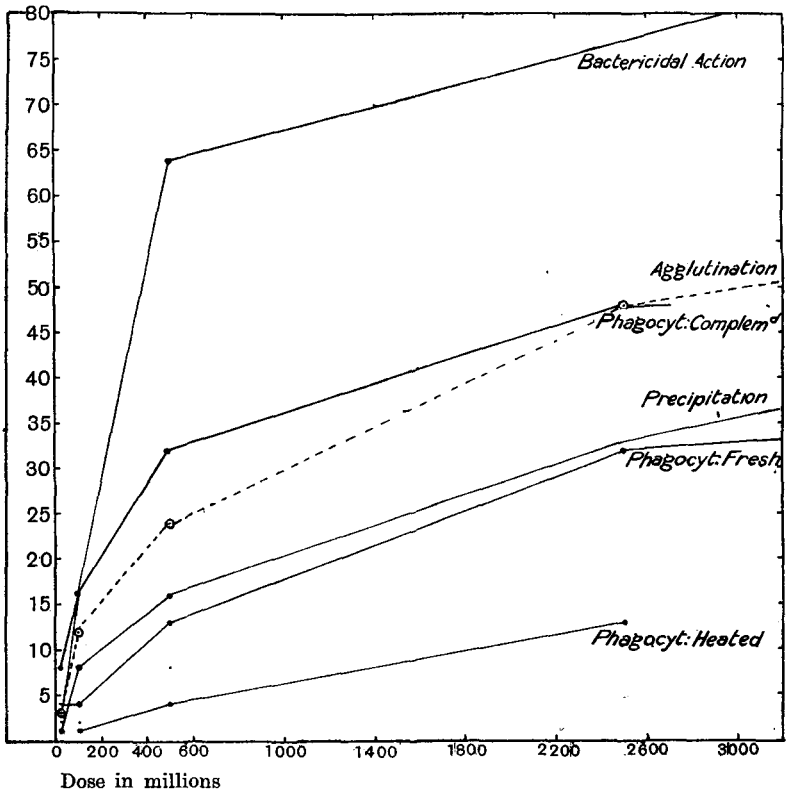
the maximum is reached sooner with the larger doses than with the smaller, and sooner with the largest than with any of the others. We shall return to this point immediately.

2. *The relation of the response to the dose.* From the figures in the table it will be seen that the maximal response in general increases with increasing dose. In the cases of agglutination and phagocytosis this increase proceeds uninterruptedly and apparently fairly regularly from the lowest dose to the highest, but in the columns for "precipitation" and bactericidal action the sequence is broken by the results of the 2500 million injections. From what has been already said the fall of the precipitin value with this dose may be due simply to a change in the extract with which the samples of this series were tested, and the fact that it is in this same series that the fall of bactericidal power occurs, though curious, may be simply coincidence. The low value of the bactericidal action, however, is not due to an error of this kind. We repeated the examination of the samples of this series more than once, and, although for reasons already discussed the maximal value found varied a little, it never reached a figure so high as that of the 500 million series, and was always found to occur in the same sample of the series. In these tests we met with nothing of the nature of a zone phenomenon nor any indication of a Neisser-Wechsberg reaction (which we obtained in none of the series, although attention was specially directed to this point); and the smoothness of the curve (see Curve 2) is against the probability of such an explanation. Any lowering of the curve due to excess of amboceptor should disappear as the amboceptor falls again after passing the apex, and the curve would show a double peak instead of the steady fall it actually exhibits. There seems no reason to doubt that the figures given represent with fair accuracy the actual value of this series relatively to the others, if the method of testing is valid at all, and that a less reaction followed this injection than followed a higher or lower dose.

The explanation of this irregularity is not obvious. We had anticipated that such a fall in the serum-values might be obtained with high doses of vaccine, if we reached that limit to the response which, as we saw on p. 77, there is reason to expect. The result of the highest dose, however, shows that this limit had not been reached, and the large difference between the two top doses (2500 and 12,500 millions) makes it improbable that the low reaction is a suggestion that we were already approaching that limit with the lower dose. It is possible that in this batch of 10 rabbits chance had brought together an unusual

proportion of animals with low reactive power, though the results of the agglutination and phagocytosis experiments do not support this view. Whether it is really a chance result or has some actual significance could be satisfactorily settled only by repetition of the experiments.

In Curve 5 the results are plotted on a diagram which brings out the relationship between dose and effect more clearly than the table. (The irregular values in the "precipitin" and bactericidal series are

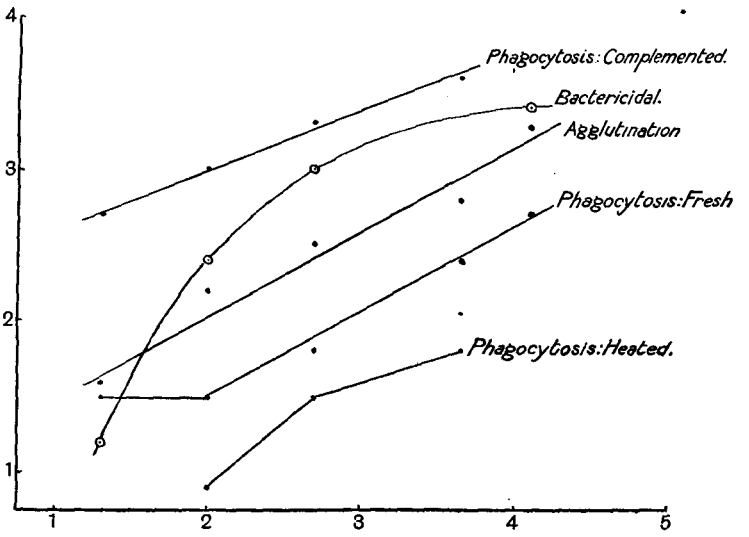


Curve 5. The maximum response is plotted against the dose. The scale is reduced and altered for the different antibodies to bring them all within one diagram.

omitted.) It appears from this that while the effect is still increasing with the increase of dose, relatively it gets less and less with each increase. The course of the individual curves clearly suggests that if we extended our range of dosage considerably but not indefinitely higher, we should reach a point beyond which a further increase of

dose would produce no further increase in response, *i.e.* we should reach the anticipated limit.

In Curve 6 these same results are plotted in a different way, which illustrates also a difference between the bactericidal and the other functions investigated. Here the logarithms of the serum-values are plotted against the logarithms of the doses. From the nature of our data, in which the value of the various samples was for the most part determined with only a rough approximation to the real value, we cannot expect to deduce with certainty any general mathematical expression correlating the dose with the effect, nor to find any very good agreement between calculated and observed values, if such an



Curve 6.

expression were found. It will be seen from this figure, however, that the curves of the various antibodies (with exception of bactericidal action) tend to take the form of straight lines, and do so within the range of error of our method. If for each antibody we calculate the values according to the formula Effect $= K \times (\text{Dose})^n$, when by "effect" we mean the maximal effective dilution and K and n are constants, the value of K is really not far from constancy. For example, with $n = 0{\cdot}43$ the values of K differ by less than $0{\cdot}5$ % for the complemented phagocytosis figures, and with $n = 0{\cdot}55$ by 30 % for the agglutination figures. But if we take "effect" as representing the amount adsorbed and "dose" as representing original concentration of the antigen, this

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is a form of the well-known adsorption-formula, and we have the relationship between dose and effect following the rules of adsorption phenomena.

This result is not so improbable as it might at first sight appear. The figures in our tables indicate the dilution in which the serum was effective. They accordingly express directly the strength of the serum, and this varies directly with the concentration of the antibody. Now it is reasonable to suppose that the greater the stimulus to antibody production (using stimulus in its widest sense) which the cells experience, the greater within limits will that production be. In measuring the strength of the serum, then, we are indirectly measuring the stimulus applied to the cells. But this stimulus is supplied by the antigen taken up by the cells, and we have therefore in the strength of the serum an expression of the amount of antigen taken up by the cells—*i.e.* the amount bound. This quantity itself depends on the quantity of antigen actually offered to the cells, *i.e.* upon the injected dose or original concentration, and it is not at all impossible that the taking up of antigen by the cells of the tissues from the body-fluids is an adsorption process. If so, then the relationship between the amount adsorbed and the original concentration will be as stated in the above scale, when the amount of adsorbing substance, *i.e.* the cells, is kept constant, as in our experiments it may be taken to be. Accordingly, since the measure of the antibody produced is an indirect measure of the amount of antigen bound, we might expect the relationship of $\text{Effect} = K \times (\text{Dose})^n$ to hold good. The process may be compared to a haemolytic experiment in a test tube, if we assume for the moment that the taking up of the lysin by the cells is an adsorption process. In this case we might measure the amount bound indirectly by the effect produced, viz. the haemolysis, instead of directly by determining the amount left free. In the case of antibody-production we measure in a quite similar way the amount bound by the effect produced, viz. the antibody formed, the animal body taking the place of the test tube and the cell of the tissues the place of the red corpuscles.

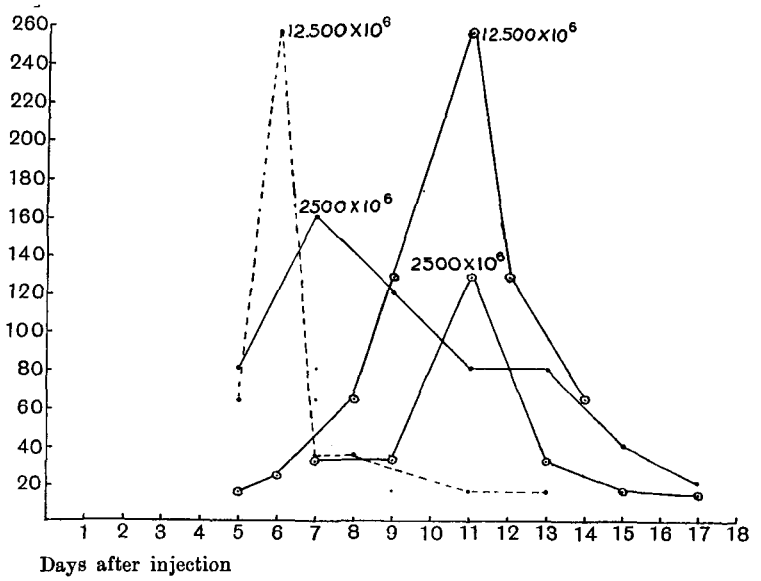
Our data then may be taken as in some degree suggesting that the process of fixing antigen by the tissue-cells is an adsorption process. They are unfortunately not sufficiently precise to do much more than suggest it: and the figures may admit of other interpretations. It would appear possible, however, to arrive in this way at some idea of the nature of certain processes occurring within the animal body, or at least of the rules which govern them.

3. *The relation of the antibodies to one another.* It has already been pointed out, when speaking of the date at which the maximum serum-value was reached, that in this respect the bactericidal action differs from the other antibodies investigated. Instead of the date getting later as the dose is increased, the bactericidal maximum comes earlier with increasing dose, and the difference is so decided and so consistent throughout the series that it appears impossible to attribute it to merely accidental circumstances. A similar distinction of the bactericidal function is met with in the relation which its maximum response bears to the dose. In Curve 6 it is evident that while the plotting of the other antibodies gives lines which do not vary greatly from straight lines, the plotting of the bactericidal values gives a line which is quite definitely curved. These differences point to a real distinction between the bactericidal function and the others, and without going into a detailed discussion of the subject something must be said of their bearing on the question of the identity of the various antibodies.

As soon as it was shown that after immunisation with a single antigen the serum of the treated animal had acquired not one only but two or more new properties, the question arose at once whether each property had a distinct substance corresponding to it or whether there was only one single substance which under different conditions acted in different ways. As all attempts have as yet failed to answer this question by direct means, such as isolation of different substances carrying the characteristic properties, the problem has been attacked indirectly, and our data supply an excellent example of the kind of evidence on which reliance is usually placed. In Curve 7 are charted the bactericidal and phagocytic values of all the samples after the injection of the two largest doses of vaccine. It will be seen that the curves of the two functions show no sort of agreement for either of these doses. In both cases the bactericidal action comes to a maximum some days before the phagocytic action and has fallen again to a relatively low figure while the other is still rising. (A similar disagreement occurs with the low doses, but in these it is the phagocytic action that is falling while the bactericidal is rising. With the middle doses the curves are not so dissimilar, the maximum in both cases falling on the same day; and this fact, while illustrating the unreliability of arguments based upon an apparent agreement, may explain the results of those who, like G. Dean (1907), have found the two functions to vary together.) If these curves represent the antibody-content of

the serum with anything approaching accuracy, it seems hardly possible to avoid the conclusion that the two functions are due to different substances. It is on evidence of this kind that the identity of two antibodies is denied, not only in the case of bactericidal and phagocytic action but for most of the known antibodies.

The validity of the evidence manifestly depends on the condition stated, that the points on the curves do represent with something like accuracy the antibody-content of the different samples of serum. They need not, however, represent this content absolutely. It has recently



Curve 7. The curves with maxima on the 6th and 7th days are those of bactericidal action; the curves with maxima on the 11th day are those of phagocytosis with fresh serum after the corresponding injections. (Scales reduced.)

been very clearly shown by H. R. Dean (*loc. cit.*) in the case of precipitin and complement-fixation reactions, that a single simple testing gives no accurate measure of the absolute capacity of a serum, and this fact is recognised by most persons familiar with the titration of haemolytic sera. Probably in no case up to now has the absolute content of a serum been accurately determined for any antibody, not even for diphtheria antitoxin. The effect produced is so dependent on the manner of obtaining it (cf. the Danysz phenomenon) that it may be made to vary in different circumstances within limits which are

quite undefined. It is sufficient, however, for the argument as to identity that the values of different sera shall be determined with fair accuracy relatively to one another.

Now in the case of the two functions shown on our chart there is an obvious difference in the method of obtaining the results, which may at first sight make comparison seem invalid. The bactericidal values are obtained upon a titration of the serum with the bacteria which lasted for four hours; in the phagocytic methods only 10 minutes were allowed for the process. In the former the action was nearly at an end, in the latter it was interrupted before it was nearly complete. It is not unusual in immunity for a reaction to run, at least for a time, more quickly with a certain dose of serum than it does with doses higher or lower. In a haemolytic series, for example, it may sometimes be seen that one or two tubes show more advanced lysis after, say, 15 minutes than the tubes above or below it, while the final results show no irregularity: and similar phenomena are known to occur with ferments. It is conceivable that in taking different times for our two reactions this may be misleading us here. We do not, however, think that this objection is really sound. The bactericidal action is practically over, and if an error of the kind indicated is taking place, it would lie in the short-time phagocytic series. Apart from the fact that the phenomenon has not, so far as we know, been observed in phagocytosis, to explain the different course of the curves on this ground would mean that the displacement of the maximum occurred regularly and progressively in each of the successive samples from the 6th day to the 11th, that it occurred similarly and regularly in all three forms of the phagocytosis tests in spite of their different character, and it would further involve a quite remarkable raising of the true maximum above the apparent one, already surprisingly high. Moreover the objection does not apply to the long-time agglutination tests, yet here the date of the maximum value in the different series agrees with the phagocytosis date and not with the bactericidal date.

A criticism of another kind may be brought forward on lines indicated by H. R. Dean (*loc. cit.*). Dean, working with precipitation and complement-fixation, showed that in these cases to every dose of antiserum there corresponds a dose of antigen with which a maximum reaction can be obtained. This dose of antigen is not inversely proportional to the dose of antiserum but may, at least within certain limits, fall as the antiserum falls. It follows from this that when equal volumes of two sera of different strengths are tested against a fixed

quantity of antigen, the resulting action in the two cases will depend on the quantity of antigen selected. This may chance to be chosen so that it is the optimal or nearly the optimal amount for the weaker serum, which therefore gives nearly its maximum reaction. It is in that case not the optimal amount for the stronger serum, and the maximum reaction possible for this serum is accordingly not obtained. The reaction got may in fact be actually less than that got with the weak serum, which therefore appears to be the stronger.

It also follows from the same fact that the method of titrating a constant and equal volume of the two sera against amounts of antigen which are reduced until a desired effect is produced (the method hitherto usual in precipitin experiments) can give no immediate measure of the relative values of the two sera. For as the result of such an experiment we obtain only two isolated facts which cannot be immediately compared with one another. We learn that with a fixed but unknown quantity of antibody contained in a measured volume of serum A a certain amount of antigen is required to produce a desired effect, and that with a fixed and also unknown but different amount of antibody in the same volume of serum B a different amount of antigen is required to obtain the same effect. But no conclusion can be drawn from the difference in the amounts of antigen required as to the relative strengths of the two sera: for neither the quantity of antigen nor the quantity of antibody is equal and constant in the two halves of the experiment, and there is no basis of comparison at all.

This objection loses its force when the more usual method is adopted of keeping the quantity of antigen constant and equal in both series, while the sera are progressively diluted. If with a fixed quantity of antigen there is one quantity of antibody, and one only, which gives a maximal reaction, it is necessarily true that in a series of successive dilutions of the two sera some one of these dilutions will in each series contain this optimal amount of antibody or nearly so (unless indeed one or both sera are so weak as not to contain it even undiluted, or unless the mere act of dilution in itself alters the condition of the antibody—a contingency not at all unlikely but which has still to be proved). With this optimal dilution the maximum reaction will be obtained in both series, and will be nearly the same in both series. But, since in order to arrive at this optimal amount less dilution of the weak serum will be necessary than of the strong, the maximum reaction will occur higher up in the series of the weak serum than in the series of the strong serum, which will therefore not only be but will be shown

to be the stronger. This will hold good for any dose of antigen. The larger this dose, the larger may become the necessary amount of antiserum, but the stronger serum will still appear the stronger. In the extreme case of a relatively very large dose of antigen, the weaker serum may be so weak as not to contain even when undiluted the requisite amount of antibody (in the volume chosen). We shall then not obtain a fair measure of the relative strengths of the two, but it will still be apparent which is the stronger.

It is not even necessary in practice to determine the dose of serum which in the two cases gives the actual maximum reaction possible. A selected reaction-result of less intensity is sufficient if care is taken to exclude a possible source of error. With a fixed quantity of antigen it may no longer be true to assume that there is one and only one quantity of antibody which will give the selected reaction, if this is less than the possible maximum. There may be two such quantities, one above and the other below the quantity of antibody required for maximal reaction. One must ensure that one does not determine for the one serum this supramaximal and for the other the submaximal quantity, and this is effected by sufficient extension of the serial dilutions below the maximal point.

The results of our testing may therefore be held to give an approximately accurate indication of the relative strengths of our serum-samples, even if it should eventually be proved that the relationship between antigen and antibody, which Dean has found for precipitating and complement-fixation reactions, holds good for other antibodies also. There seems to be at present no sufficient reason for doubting that a rise or fall in our curves does represent a rise or fall of the corresponding antibodies in the serum-samples, and that the bactericidal function should in the meantime be considered as due to the action of some other substance than the phagocytic function.

Although there is more or less agreement that a greater production of antibodies is obtained from a greater injection of antigen, we have found little in the literature beyond the opsonic evidence already referred to, which bears on the question of variation in the period at which the maximum is reached. Such evidence as there is hardly supplies suitable material for comparison with that brought forward in this paper, partly because most workers have used single animals and partly because they used living organisms measured in terms of broth-cultures or of standard-loops. From the series of papers by Leishman and his

colleagues, however, there can be extracted an indication of the date of the maximum reached after the injection of 20 millions of a typhoid vaccine. They used pooled rabbit sera (two or three animals) and accurately standardised doses. Unfortunately for our purpose, however, these small injections were always followed by a second larger dose and it is rarely possible to determine accurately from their charts when the maximum point after the first injection is reached. Further, it is not always apparent whether they were using broth-cultures or emulsions from agar: and the difference may make an important difference in the result. On the whole, however, it appears as if the summit of the curve after injection of 20 millions or 40 millions came later than in our experiments, about the 10th or 11th day, or perhaps later in the case of agglutination.

It is to be remembered in comparing results of such experiments that the strain used must make a distinct difference in the effect produced. This effect depends, *ceteris paribus*, on the quantity of antigen injected. But there is no reason to suppose that, say, 20 millions of one strain of typhoid bacilli contains the same amount of antigen as 20 millions of another strain, whether equally virulent in the living condition or not; and the accumulating evidence of other small differences in different strains of varying history suggests that this is unlikely to be the case. There is in fact a current belief that different strains of organisms vary considerably in antibody-producing power. The varying degree of resistance in different strains will also affect the readiness with which the antigen is dissolved out of the bacterial bodies. We should accordingly not expect with another strain of typhoid to obtain figures either for date or value, which were the same as those in the above tables; although it would seem probable that for any one strain the increase and the delay will be found to occur with increasing quantity of antigen.

Summary.

Following the subcutaneous injection of typhoid vaccine into rabbits the average antibody response was estimated from day to day, and found to vary for agglutination, bactericidal action, phagocytosis (including tropins), "precipitation" and probably for complement-fixation according to the dose of vaccine.

As estimated by the maximal value reached by the serum the response increases with increasing dose in nearly all cases. This increase becomes relatively less and less as the dose increases, and its progress suggests

that with a sufficiently large dose a limiting-value might be reached, which if not actually a true limit would be practically one.

The observed relationship between dose and effect is such as to suggest that the combination of antigen and reacting-cell takes place in accordance with the rules governing adsorption processes; but this does not hold for the response in bactericidal power.

With increasing vaccine-dose the date at which the maximal value is reached gets later for the various antibodies, with again exception of the bactericidal power.

The precipitation observed differs from the usual precipitation reaction and is perhaps really an agglutination-phenomenon.

Phagocytosis was observed in three ways, and the bearing of the results on the mechanism of the action of the antibody concerned is considered; and the relationship between the bactericidal and other antibodies is also discussed.

REFERENCES.

ASCHER (1901). *Centralbl. für Bakteriologie*, Orig. XXIX, p. 125.

COLE (1904). *Zeitschr. für Hygiene*, XLVI, p. 371.

COWIE and CHAPIN (1907). *Journ. Medical Research*, XVII, pp. 57, 95, 213.

DEAN, G. (1907). *Proc. Roy. Soc.* B, LXXIX, p. 399; *British Medical Journ.* 1907, Nov. 16.

DEAN, H. R. (1911). *Proc. Roy. Soc. of Medicine*, VOL. V, Sect. Pathology, p. 62.

FRIEDBERGER (1902). *Festschrift v. von Leyden*, II, cit. ap. Kolle und Wassermann, IV, p. 512.

KLIEN (1907). *Johns Hopkins Hosp. Bulletin*, XVIII, p. 245.

LEISHMAN, HARRISON, GRATTAN and ARCHIBALD (1908). *Journ. Roy. Army Medical Corps*, X, p. 586; Harrison, *ibid.* VIII, p. 480; Leishman, Harrison, Grattan, Webb and Kennedy, *ibid.* XI, p. 327.

STÄUBLI (1904). *Centralbl. für Bakteriologie*, Orig. XXXVI, p. 297.

WRIGHT (1909). *Studies in Immunisation*, London, p. 337.