AN EXPERIMENTAL STUDY OF PNEUMOCOCCAL SEPTICAEMIA AND ANTI-PNEUMOCOCCAL IMMUNITY.

by

Hedley D. Wright,
B.A.(Tas.), M.D.(Edin.), M.R.C.P.Ed.
Lecturer in Bacteriology in the Medical School,
University College Hospital, London.

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Table of Contents.

The object of the investigation recorded herein has been to enquire into the mechanism by which pneumococci are removed from the blood stream of the rabbit after intravenous injection and to see how far this process is modified by immunization.

It has seemed most convenient to divide the text into two parts. In the first will be described at length the experiments that have been made, reference to the literature being made only where necessary to elucidate the point discussed: in the second part these experimental results are discussed and compared with those reported by other workers.

The literature upon this subject is very extensive and widely scattered. An attempt has been made to study all papers that one has been able to find but reference is made only to those which have contributed something which has seemed germane to the problems under discussion.

METHODS.

The following method has been employed throughout the investigation. The inoculum has been a measured amount of a 24-hour growth of a strain of pneumococcus (Type I.) in broth containing 10% to 20% of rabbit serum. The stock culture has been maintained in blood broth (50% defibrinated

rabbit's blood). At first the organism was incubated in this medium for 24 hours and then the culture was placed in the refrigerator till required. Later however it has appeared that virulence is better maintained if the inoculated, but unincubated medium is kept in the ice box and incubated when required. The bacterial content of the inoculum has been determined by plate cultures from dilutions, the diluent used being normal saline containing gelatin and sodium phosphate as suggested by Robertson, Sia & Woo (1924) to obviate the destructive effect of sodium chloride upon the pneumococcus.

Rabbits have been used for all the experiments recorded.

The concentration of the organisms in the blood of the animal at the time of inoculation cannot be accurately determined because of the rapidity with which they are removed. In all cases this figure has been obtained by calculation from the number of organisms injected and the blood volume, taken as five per cent of the body weight. All other figures have been obtained by drawing blood from the marginal vein into a small amount of concentrated solution of sodium citrate and making plate cultures from appropriate dilutions. In all cases where the blood has required dilution citrated rabbit blood has been added to each agar plate. Counts may be made with much greater facility after 48 hours' incubation than after 24 hours'.

Bull employed heart puncture for the purpose of obtaining blood for similar experiments. Some observations showed that the figures obtained using heart blood were sensibly the same as those when the blood was drawn from the ear. But there were certain cases in which the injury produced by the heart puncture occasioned or favoured the production of pericarditis and interfered with the experiment.

The accuracy of the counts obtained has been controlled as far as possible. In the case of the cultures inoculated the figures represent the average of three plates,

In the case of cultures from the blood the use of a series of three dilutions permitted gross inconsistencies to be detected. As a general rule plates containing from 100 to 1000 colonies have been more trustworthy than those containing numbers outside these limits; further the larger the bulk of the inoculum the better have been the results. With all precautions taken it seems clear that reasonably accurate results may be obtained but that differences, in order to be significant, must be relatively large.

The results are recorded for the most part in tabular form but in some cases in the form of graphs in which the ordinates represent the logarithm of the number of pneumococci per cubic centimetre while the abscissae represent time in hours or days. The use of logarithms has the disadvantage that it appears to minimise the preliminary drop in the number of organisms while it tends to

exaggerate the succeding phases. It has however the distinct advantage of permitting all the features to be recorded within a manageable compass.

REMOVAL OF PNEUMOCOCCI FROM BLOOD OF NORMAL RABBIT AFTER INTRAVENOUS INJECTION.

In chart I. (p.13) two curves will be found which illustrate the course of the septicaemia in rabbits following the intravenous injection of a non-lethal dose of pneumococci. Rabbits 177 and 178 received 1 c.c. of culture, the injections being made on different days and with different cultures of the same strain. The actual figures recorded were as follows:-

TABLE 1.

After injection.	Organisms per c.c. of blood. 177	178
Immediately	2,000,000	2,500,000
10 mins.	650,000	1,370,000
30 "	400,000	
2 hrs.	43,000	
5 hrs.	2,100	6,400
24 hrs.	64,000	16,300
48 hrs.	77,000	2,000
72 hrs.	60	30
96 hrs.	52	0
144 hrs.	0	

These curves agree in showing

(1) the preliminary period of removal lasting 5 hours;

(2) the period during which the bacteria increase in numbers;

(3) the final and more gradual removal of the organisms .

A more detailed study of the preliminary period shows that in these cases the bacteria in the blood do actually reach a minimum at 5 hours after inoculation the next counts at 6 and 7 hours showing a slight but progressive rise.

TABLE 2.

Rabit 325.	
	Organisms per c.c. of blood.
Immediate	660,000
1 hr.	163,000
4 hrs.	33,800
5 hrs.	28,500
6 hrs.	33,800
7 hrs.	47,500
8 hrs.	52,400
9 hrs.	116,000
12 hrs.	608,000

For a very long time no exception was found to this statement even in cases where the animals died within 24 hours

after inoculation. But finally cases did occur in which the count at 5 hours was as high as that at the time of inoculation. The matter was therefore reinvestigated and it was found that in certain cases the increase in numbers occurred at a much earlier period. The following figures will illustrate this point:-

TABLE 3.

Organisms per c.c. of blood			
Rabbit	432	434	354
Immediate	385,000	1,580,000	3,000,000
1 hr.	86,000	370,000	1,100,000
3 hrs.	136,000	220,000	230,000
5 hrs.	630,000	670,000	610,000

In this experiment the increase apparently began between 1 hour and three hours after inoculation in one case and in the others between 3 and 5 hours. In all cases the major part of the removal is carried out during the first two hours; after that period occurs in most cases a further slower diminution in the numbers but they may remain steady or actually increase. The exact course of events seems to depend upon the condition of the culture used and upon certain peculiarieties of the individual rabbits. That the size of dose has little effect upon this part of the curve will be seen from the following experiment. Six rabbits of varying

weight were inoculated with varying doses of the same culture and their blood examined at intervals. The number of organisms found is recorded in Table 4.

TABLE 4.

Rabbit	431	432	433	434	435	354
Weight	1,500	1,400	1,720	1,700	1,800	1,800
Dose	$\frac{1}{10}$ c.c.	$\frac{1}{10}$ c.c.	$\frac{1}{2}$ c.c.	$\frac{1}{2}$ c.c.	1 c.c.	1 c.c.
	No. of organisms per c.c. of blood					
Immediate	360,000	385,000	1,570,000	1,530,000	3,000,000	3,000,000
1 hr.	44,000	<u>86,000</u>	360,000	370,000	660,000	1,100,000
3 hrs.	7,200	136,000	10,600	<u>220,000</u>	130,000	<u>230,000</u>
5 hrs.	<u>4,900</u>	630,000	<u>3,200</u>	670,000	<u>70,000</u>	610,000
24 hrs.	5,280,000	+	6,300	+	2,480,000	3,030,000

Figure underlined = smallest number recorded.

It is clear that the minimum number of organisms attained in the first five hours and the time at which the increase in numbers occurs bears no direct relationship to the dose injected. Further in the rabbits inoculated with similar doses of pneumococci the result may vary very considerably in a way which can only be referred to the peculiarities of the individual animal. In spite of this observation it has seemed advisable in experiments where comparisons are being made to use animals of about the same weight. This has not always been possible but it has not appeared that there is any serious

disadvantage attached to the use of animals differing in weight by as much as 500 grams. The lighter animal (1,500 grams) has differed very little from one of 2,000 grams.

The condition of the particular culture employed has a very marked effect upon the early part of the curve. As a general rule if the animal dies quickly it will be found that the preliminary clearing is less marked and of shorter duration but this is not always the same. If the organism is allowed to grow under conditions which do not favour the maintenance of virulence the preliminary removal becomes much more effective and may be complete. This strain of pneumococcus was grown for a period of some two months, with daily subcultures, upon a phosphate broth medium containing glucose but no serum. At the end of this time it showed a slight tendency to become granular in the broth culture, unlike the ordinary culture in this medium which is quite evenly dispersed. Such a culture which was quite avirulent for rabbits was inoculated intravenously in a dose of 1 c.c. into each of two rabbits with the results given below.

TABLE 5.

Organisms per c.c.		
Rabbit	349	350
Immediate	8,900,000	7,100,000
30 mins.	4,800	294
2 hrs.	206	18
5 hrs.	20	2
24 hrs.	0	0

Here the removal of the organisms was almost immediate and was very nearly complete in five hours.

It has not been possible to predict the course of the second part of the curve from what has happened in the first few hours. As a rule the number of organisms present in the blood after 24 hours is greater than that at the end of the 5-hour period, and there appears to be some relationship between the figures. If it is low at five hours it is usually also relatively low at 24 hours; if high at 5 it is correspondingly high at 24. But there are very many exceptions. Sometimes the increase is very great during this period but in other cases it is slight or the number remains about the same, while in still others an actual diminution occurs. The rate at which increase occurs during this period appears to depend on some factor or factors within the animal as it varies from animal to animal in a series inoculated with

equal amounts of the same culture (see Table 4). It is also dependent upon the virulence of the culture. If the number of organisms at 24 hours is very high (hundreds of thousands or millions per c.c.) the animal usually dies within three days but if it is lower (tens of thousands or less) the rabbit frequently recovers.

The process of recovery is evidenced by a gradual disappearance of the organisms from the circulation, complete as a rule in from four to six days. The exact course of this part of the curve is subject to wide variations. Sometimes there is little change in the numbers during the second day and then a very sudden diminution occurs in the course of the third day; in other cases the process is a much steadier one. The curve may however make a second ascent which may begin at any point and go on for varying periods. This has usually meant that the animal has developed peritonitis or endocarditis, or some other local lesion. Death may occur on the fourth day or later although the actual number of organisms is considerably less than on the third day. Some fifteen or twenty animals developed iritis and ophthalmitis of varying degrees of severity, usually well marked. In these cases there seemed to be little, if any, effect produced upon the bacteriaemia.

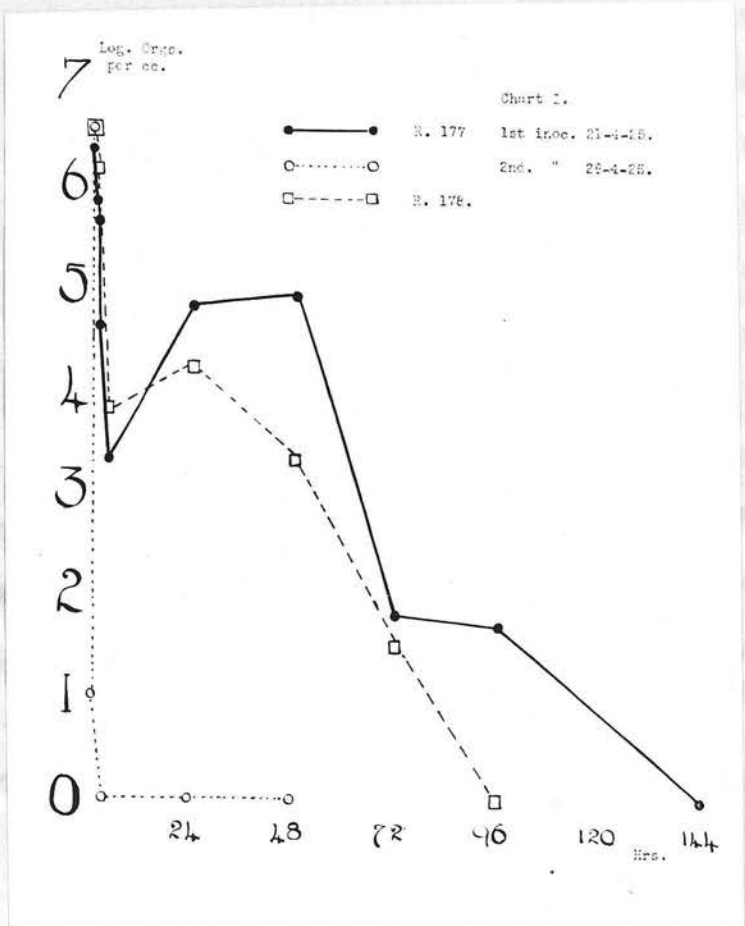
The foregoing summary of results obtained with normal animals emphasises the very great variability observed in the course of the bacteriaemia. The difficulty arising from

inherent peculiarities of individual rabbits cannot be entirely obviated in experiments involving comparison of the effect of different procedures. I have attempted to reduce them to a certain degree by including as many rabbits as can conveniently be handled - nine seems to be about the maximum number. So far as the variations in the properties of the culture are concerned exact standardization each day would have left little time for the experiments so that one has had to content oneself with using what appeared to be adequate controls.

THE EFFECT OF IMMUNIZATION UPON THE COURSE OF THE BACTERIAEMIA.

If a rabbit be given a second intravenous injection of pneumococci immediately after it has completed the removal of a similar dose from its circulation it is found that the curve is an entirely different one.

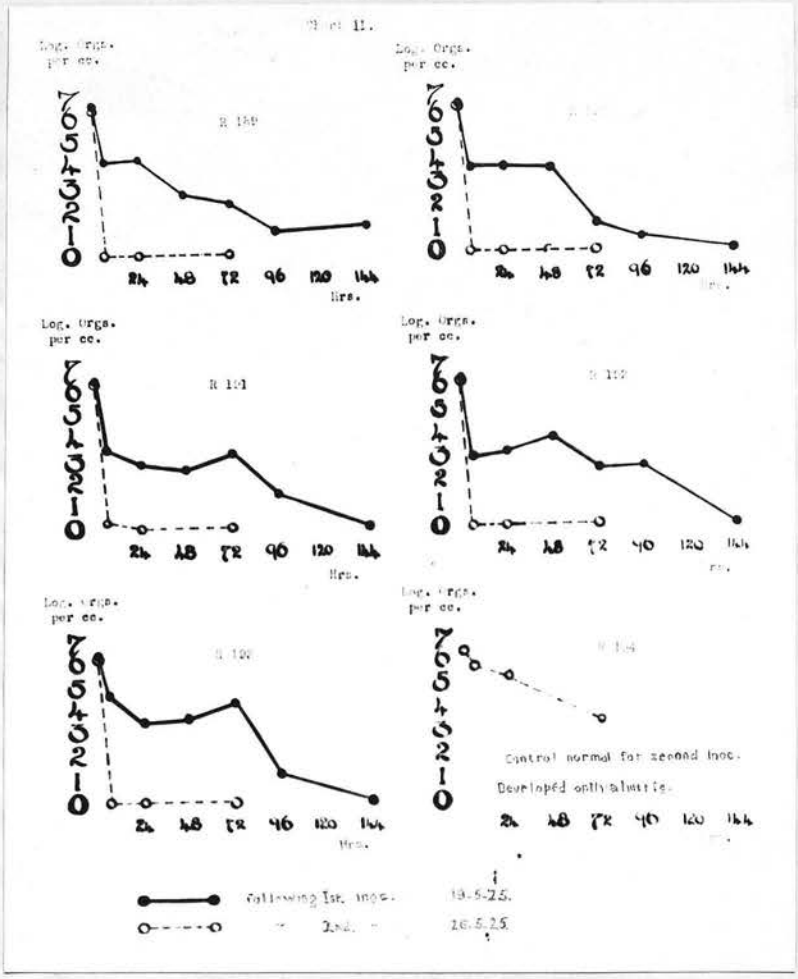
CHART I.



Rabbit 177 was given an intravenous injection of 1 c.c. of culture of pneumococci on 21.4.25. The course of the bacteriaemia is indicated by the black continuous line. The organisms had disappeared from the blood on the sixth day after the injection was made. On the next day (28.4.25) a similar dose was injected into rabbit 177 and into a normal rabbit 178. The course of the bacteriaemia in the control rabbit (broken line with squares) was similar in type to that following the first injection into rabbit 177. But in the case of rabbit 177, now an immunized animal, very few organisms (10 per c.c.) were found at the end of 10 minutes and none at the end of 5 hours. Further, the removal was permanent for no organisms could be found at the end of 24 or 48 hours. The effect of the first injection was to increase the preliminary rate of removal and, in this case, to prevent any subsequent reappearance of organisms in the blood.

Similar phenomena (see Chart II.) were observed in the case of a group of five rabbits (189 - 193) which received their first injection on 19.5.25, and the second on 26.5.25, after the first dose had been removed. The normal rabbit (194) behaved in such a way as to show that the difference observed was not due to changes in the culture.

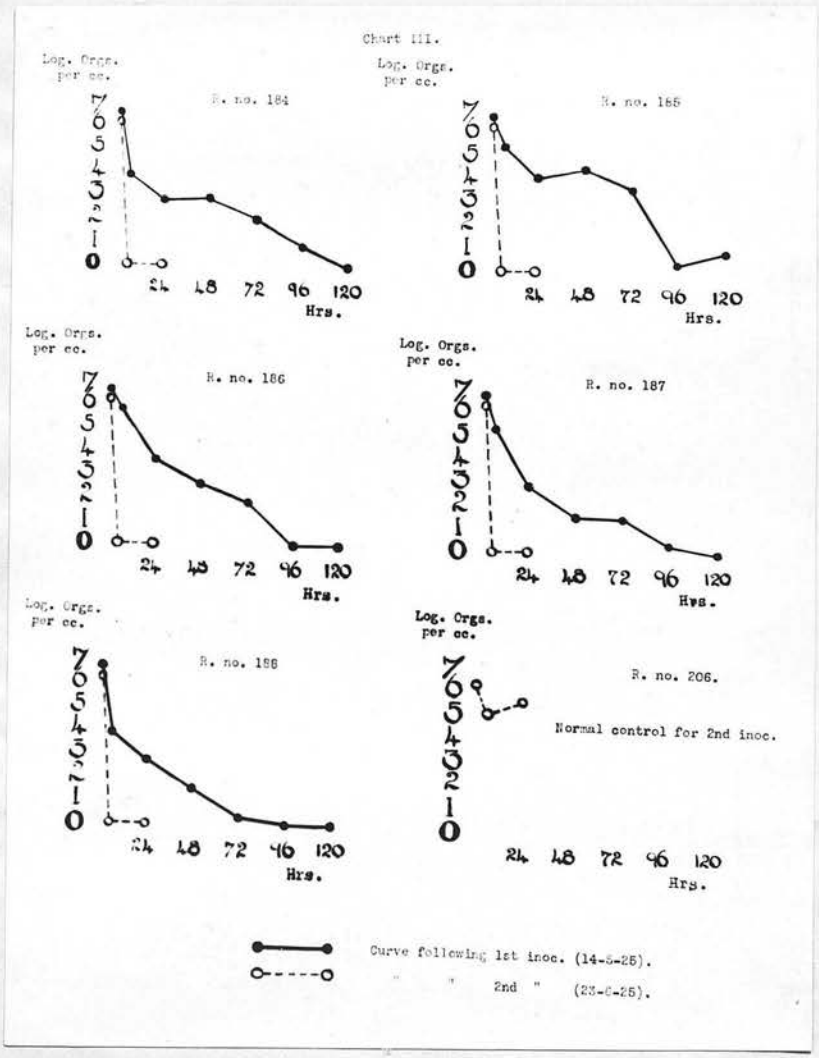
CHART II.



This very striking result was somewhat unexpected in view of the difficulty so frequently experienced in producing antibodies to the pneumococcus in rabbits. Accordingly another series of rabbits (184 - 188) which received a first injection on 14.5.25 were given a second injection on 23.6.25, five weeks after the first and about a month after they had disposed of this first injection.

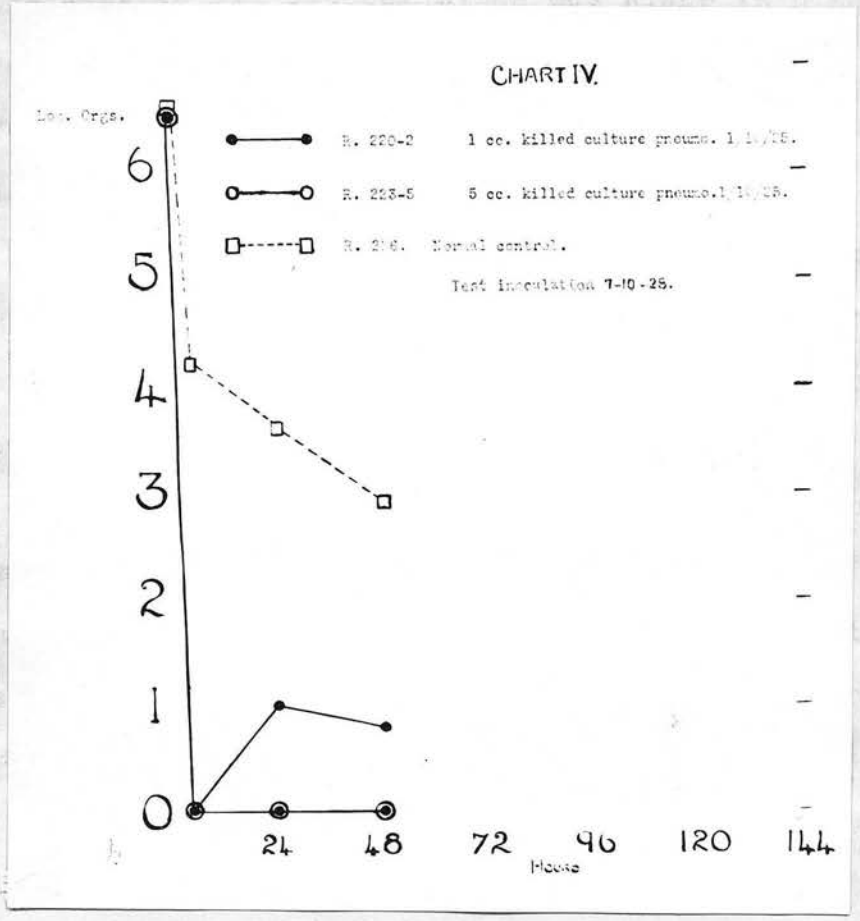
In Chart III. are recorded the results following both injections. The results are entirely concordant with those previously described. This increased capacity for clearing the pneumococci out of the circulation is retained for at least a month. The curve of the control normal rabbit (206) indicates that the culture could not be held to have altered sufficiently to account for the altered result of the inoculation.

CHART III.



The effect of the <u>preliminary injection of a killed (formalinized) culture</u> of pneumococci upon the subsequent removal of an injection of living organisms was now tested (Chart IV.)

<u>CHART IV.</u>



Rabbits 220, 221, 222 were given each 1 c.c. of killed culture intravenously, while rabbits 223, 224 and 225 each received 5 c.c. of the same culture on 1.10.25. Six days later all these animals, together with a normal control (226) were inoculated intravenously with 1 c.c. of the usual living culture. The normal animal behaved in the usual fashion. The blood of all the test animals excepting number 222 was clear of organisms in 5 hours and remained so. In the case of No. 222 the blood was clear in 5 hours but there was a return of a small number of organisms (10 per c.c.) in 24 hours and there were still 7 per c.c. present at the end of 48 hours.

It is of course impossible to test the clearing power of the same rabbit both before and after the injection of a killed culture as the effect of a first injection of living culture could not be disentangled from the effect of the subsequent injection of killed organisms. The difference between the curves of the treated and that of the untreated animals is sufficiently marked to be evidence that a suitable dose of killed culture can increase the capacity of an animal to remove organisms from its circulation.

In view of the apparently discordant results recorded in the literature the course of events during the first five hours was investigated a little more fully. The bacteriaemias produced in the previous experiments were of a low grade and all the animals recovered from their inoculations.

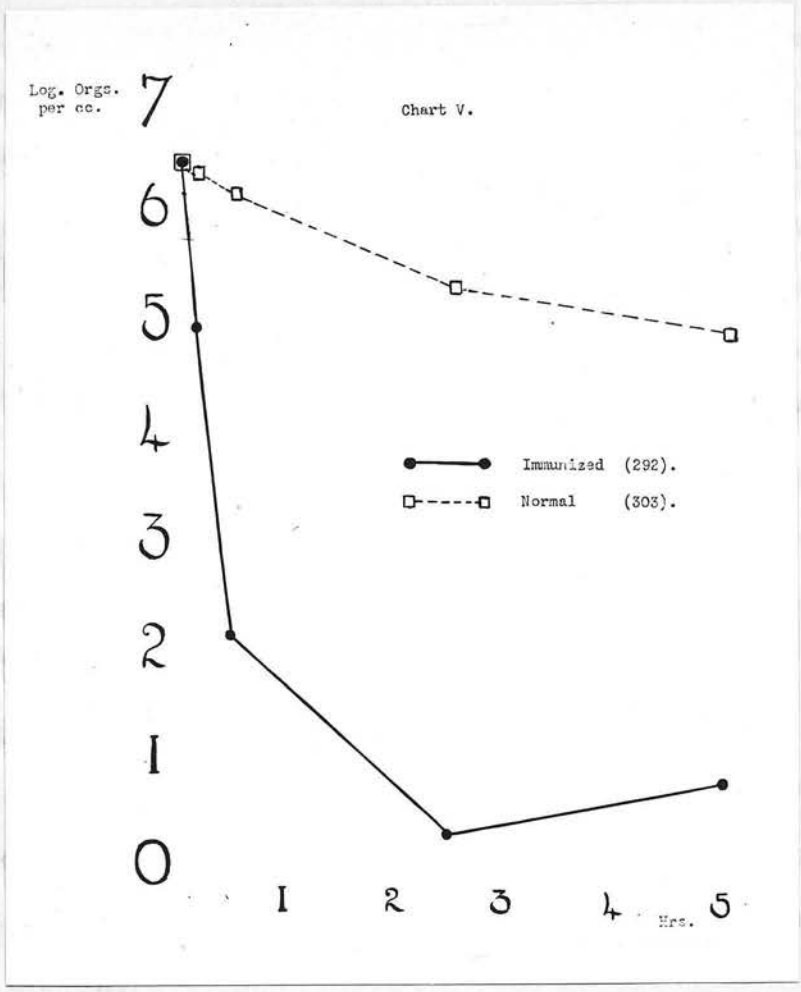
By the end of 1925 the virulence of the culture had become enhanced and the test dose of 1 c.c. proved fatal for most normal rabbits. It was used to compare the "clearing capacity" of a normal rabbit (303) with that of an animal of similar size which had been inoculated with 5 c.c. of killed culture of pneumococci 6 days previously. The figures actually observed were as follows:-

TABLE 6.

	Organisms per c.c. of blood.	
	R.292 (treated)	R.303 (normal)
Immediate	2,700,000	2,500,000
10 mins.	80,000	1,800,000
30 "	134	1,340,000
2½ hrs.	2	180,000
5 "	6	76,000
24 "	652	9,000
48 "	40	46,000
72 "	0	D i e d.

The figures for the first five hours are recorded in graphic form in Chart V.

CHART V.

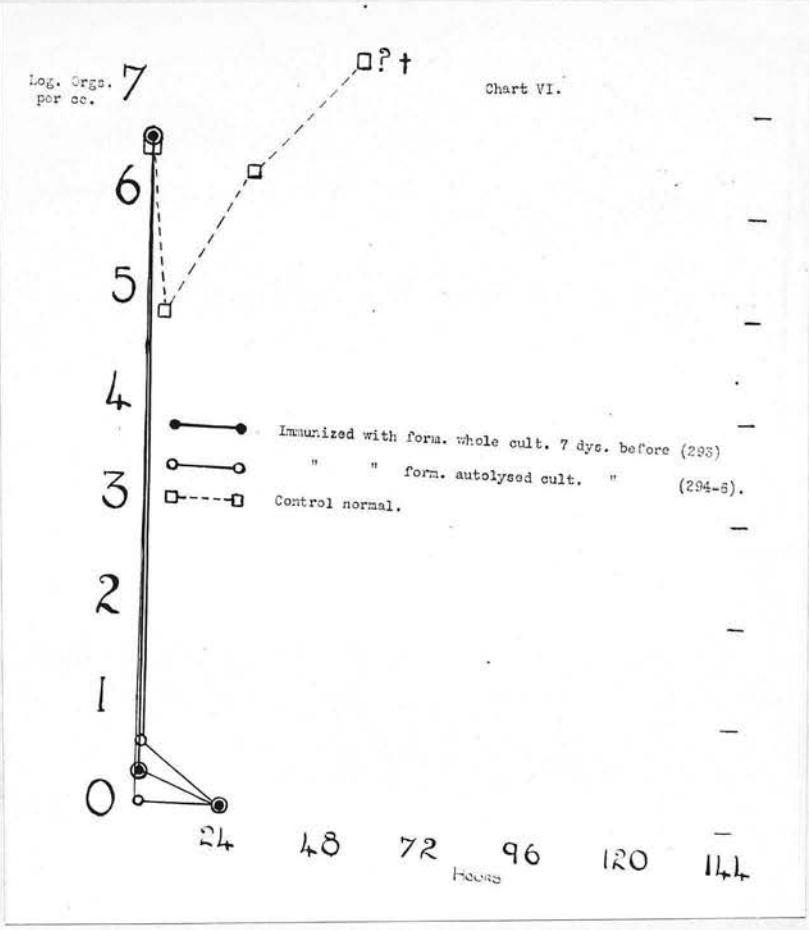


The difference in the form of the curves is very marked. The result of this experiment is also of importance in that it indicates that the precise result of an injection into an immunized animal will depend on the degree of immunization

and the virulence of the organism. Under certain circumstances the curve may be of the type found in the normal animal but at a much lower level - it differs in degree but not in kind.

Finally it was found that a similar change may be brought about by the use of cultures which have been allowed to autolyse by incubation for an extended period. A broth culture of pneumococcus (without serum) was incubated for four days until all opacity had disappeared and the clear fluid removed from a slight deposit at the bottom of the tube. This perfectly clear liquid (free from visible organisms) was inoculated intravenously into rabbits 294, 295 and 296 in a dose of 5 c.c. An equal amount of formalinized (not autolysed) culture was inoculated into rabbit 293. A week later these together with a normal control rabbit were inoculated with the test dose of living culture. The results are recorded in Chart VI. This dose which killed the control animal is practically completely removed in 5 hours and there is no subsequent return of organisms to the circulation.

CHART VI.


Log. Orgs. per cc.
7
6
5
4
3
2
1
0
□? †
Chart VI.
Immunized with forma. whole cult. 7 dys. before (293)
" " form. autolysed cult. " (294-6).
Control normal.
24
48
72
96
120
144
Hours

By any one of these procedures then, preliminary injection of living, killed or autolysed culture the capacity of an animal to remove the homologous type of pneumococcus can be considerably increased and subsequent multiplication can be prevented or diminished. Immunity may be present in experimental animals in different degrees and it appears that the particular type of curve obtained in a given experiment depends on the conditions found in that animal by the particular culture used. The difference between the curves of naturally resistant animals and those of artificially immunized ones seemsto be a matter of degree rather than kind. Within certain limits it is possible to obtain almost parallel curves from both series of animals though the cultures employed would of course be quite different. This suggests that the improvement effected by the process of immunization is of the nature of an increase of a normal function rather than the establishment of a new one, a view of antipneumococcal immunity which is also suggested by Woo (1926).

TIME AFTER INOCULATION WITH KILLED CULTURE AT WHICH THE IMPROVED CLEARING CAPACITY IS MANIFESTED.

In the experiments designed to settle this point groups of three rabbits were in each case inoculated intravenously with 5 c.c. of formalinized broth culture of pneumococci. At various periods thereafter these animals were given an intravenous injection of the test culture (1 c.c. of serum broth culture). At the same time an equal number of control normal animals was given an equal dose of the same culture by the same route. The curves of the bacteriaemia in both groups of animals were then compared.

Effect of a dose of killed culture 5 hours before the test dose. In this experiment in addition to the test and normal control animals a further group of three animals was given 5 c.c. of formalinized broth culture of B.typhosus so as to test whether any result produced was specific. All these animals suffered from severe diarrhoea, a factor which may have invalidated the figures because of the loss of fluid. The actual results obtained were as follows:-

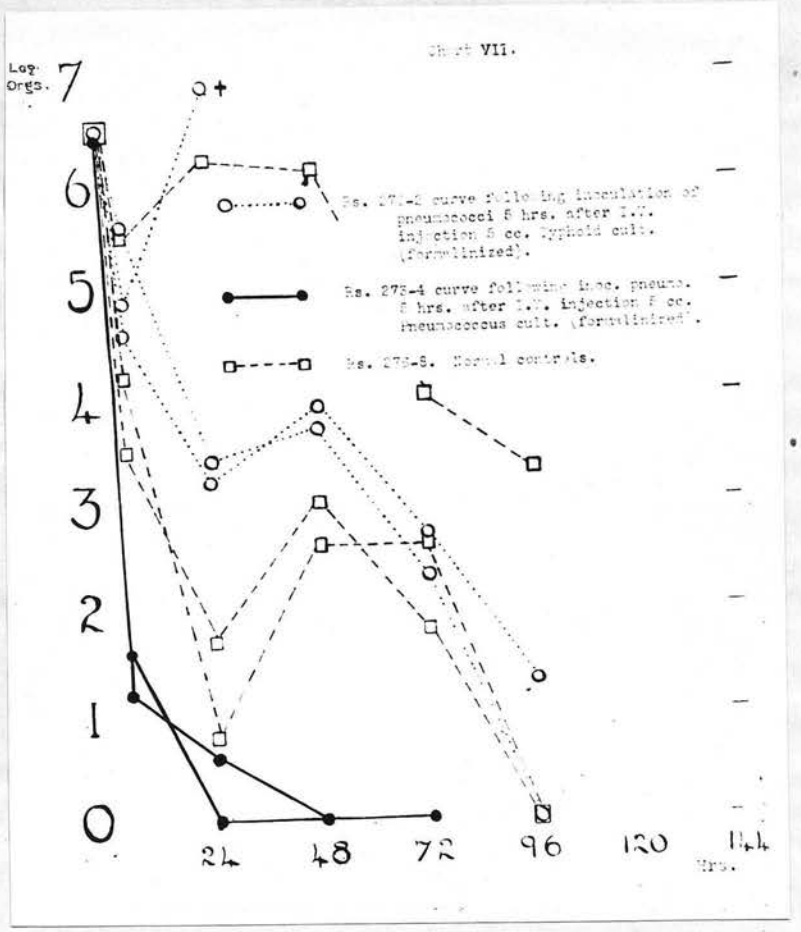
TABLE 7.

Organisms per c.c. of blood.

	Preliminary dose Typhosus.			Prelim.dose Pneumo.		Untreated.		
Rabbit	270	271	272	273	274	276	277	278
Immediate	2,900,000	3,000,000	3,200,000	2,600,000	2,700,000	2,700,000	2,900,000	3,300,000
5 hrs	41,000	78,000	41,000	16	36	17,400	3,400	309,000
24 "	2,400	8,253,000	1,600	4	0	6	48	1,617,000
48 "	5,100	Dead.	7,600	0	0	394	982	1,400,000
72 "	214		474	0	0	370	68	9,100
96 "	Killed.		18			0	0	1,800

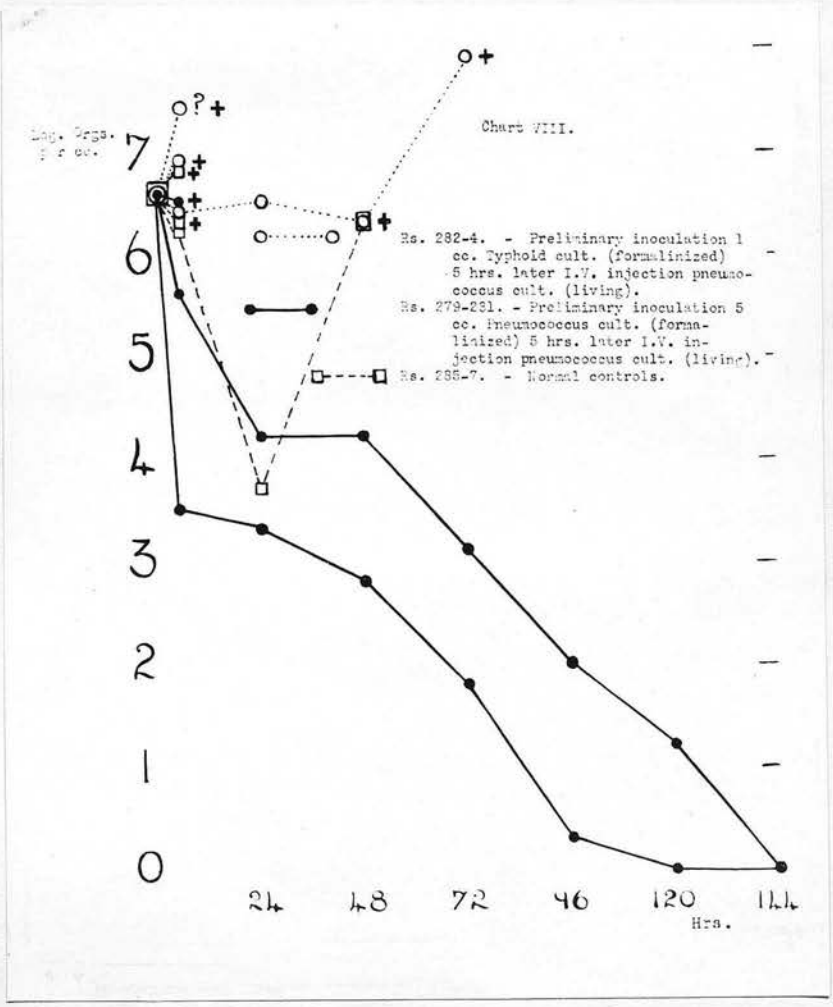
In Chart VII. these figures are recorded graphically. In this case there is a fairly well marked advantage accruing to the animals which had received the dose of pneumococci as compared both with the normals and with those treated with B.typhosus.

CHART VII.



A further experiment on the same lines but with a culture in a more virulent condition was carried out at a later date. The control animals treated with B.typhosus were this time given only 1 c.c. of killed culture. They also suffered slightly from diarrhoea. Chart VIII. shows the general result of the experiment. The only two animals which recovered were two of the three which had received killed pneumococci 5 hours before. The curves of the bacteriaemia in these animals is at a lower level than those of the others.

CHART VIII.



Still another experiment of the same kind made some time later and recorded on page 80 also indicates that treatment of this kind may in some animals produce a slight improvement in the preliminary clearing of organisms from the blood. In that case a still smaller dose of B.typhosus had a similar effect in some of the animals.

From these experiments one can conclude that under these particular conditions a slight benefit is obtained from an injection of killed organisms as early as five hours after the injection. There is no indication of the occurrence of a negative phase. The idea that such a phenomenon does occur is based upon the observation that antibodies may become diminished after such an injection. But Stevenson & Kapadia (1925) in the case of B.pestis, Teague & McWilliams (1917) in the case of typhoid injections and Morgenroth and his colleagues in the case of streptococcal and B.Gaertner infections have noted the development of slight degrees of immunity at equally early periods after inoculation. Issaeff (1894) had noticed also a non-specific increase of resistence which appeared 7 - 8 hours after inoculations of cholera vibrios.

Similar experiments 24 and 48 hours respectively after the first inoculation gave the following results:-

TABLE 8.

	Injection 24 hours after inoculation of killed culture.					
	Treated.			Normal.		
Rabbit	247	248	249	250	251	252
Immediate	3,900,000	3,900,000	3,900,000	4,000,000	3,900,000	3,750,000
5 hrs.	1,500	6,300	148	33,500	24,300	10,800
24 "	5,000	678	502	5,500	888	1,174
48 "	0	24	74	1,338	6,800	15,800
72 "	35	0	4	122	814	78
96 "	0	0	0	0	89	0
120 "	0	0	0	0	0	0

Chart IX. shows that the curves in the case of the treated animals tend to lie at a slightly lower level than in that of the normals though there is a slight amount of overlapping; the difference at the end of five hours is however fairly well marked and might be regarded as indicating a slight improvement in clearing capacity resulting from the treatment

CHART IX.

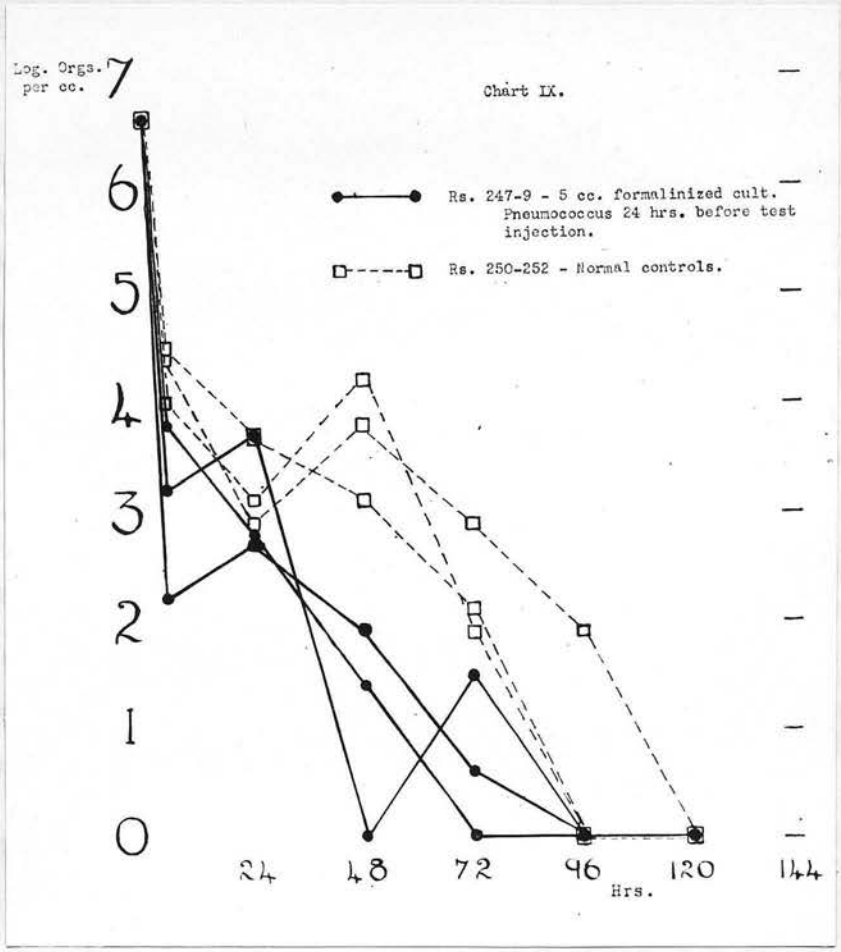

Log. Orgs. per cc.
Chart IX.
Rs. 247-9 - 5 cc. formalinized cult. Pneumococcus 24 hrs. before test injection.
Rs. 250-252 - Normal controls.
7
6
5
4
3
2
1
0
24
48
72
96
120
144
Hrs.

TABLE 9.

	Injection 48 hours after inoculation of killed culture.					
	Treated.			Normal.		
Rabbit	254	255	256	257	258	259
Immediate	3,500,000	3,200,000	3,200,000	3,900,000	3,200,000	3,200,000
5 hrs.	2,800	25,100	30,900	964,000	567,000	765,000
24 "	1,260	6,400	12,600	32,600	1,616	18,800
48 "	2	92	3,746	16,700	5,098	2,732
72 "	0	14	1,898	3,660	1,100	84
96 "	0	1	36	248	1,334	182
120 "	0	0	4	1	4	30
144 "	0	0	0	0	0	24

The graphs are recorded in Chart X.

In this case there was a very striking difference in the preliminary period of removal but the subsequent course of the curves is not markedly different in the two groups. Two of the curves of the treated animals fall distinctly below the normal zone but the third lies practically within it.

TABLE 9.

	Injection 48 hours after inoculation of killed culture.					
	Treated.			Normal.		
Rabbit	254	255	256	257	258	259
mmediate	3,500,000	3,200,000	3,200,000	3,900,000	3,200,000	3,200,000
5 hrs.	2,800	25,100	30,900	964,000	567,000	765,000
24 "	1,260	6,400	12,600	32,600	1,616	18,800
48 "	2	92	3,746	16,700	5,098	2,732
72 "	0	14	1,898	3,660	1,100	84
96 "	0	1	56	248	1,334	182
20 "	0	0	4	1	4	30
44 "	0	0	0	0	0	24

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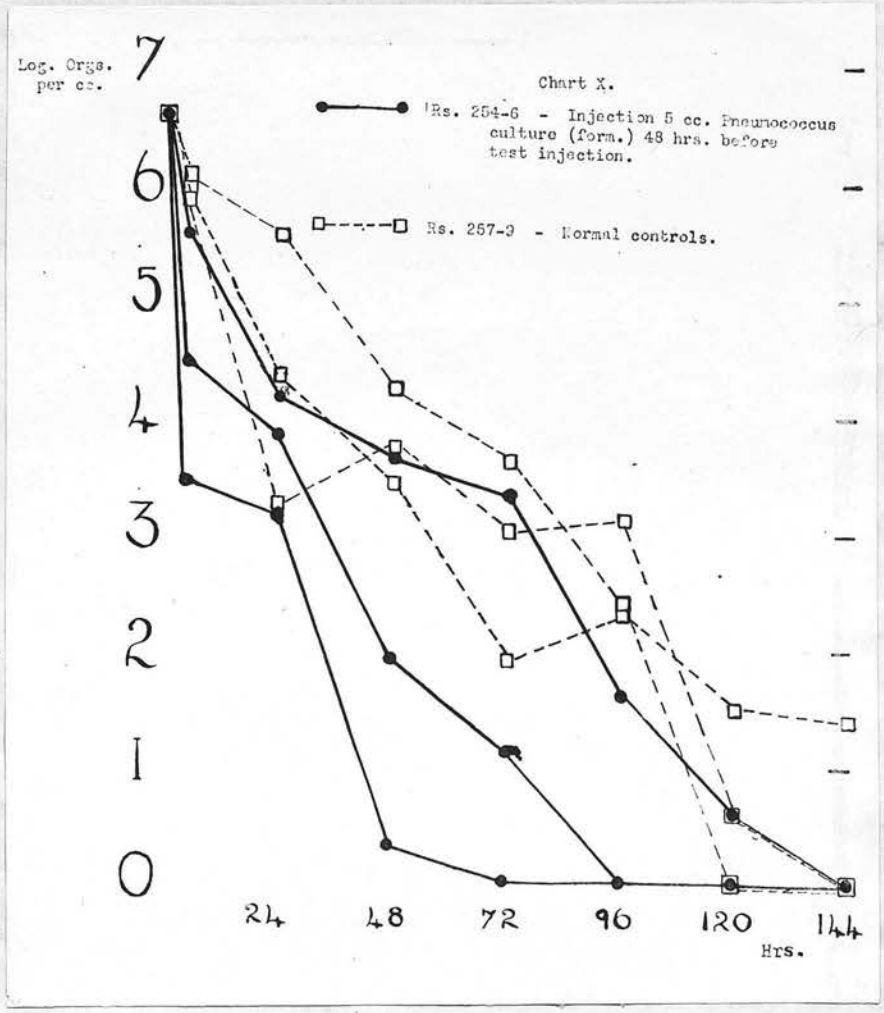
CHART X.



Up to this stage then the alteration in the type of curve is slight but appears to be all in the direction of a slight improvement in clearing capacity. The figures obtained from the experiment on the third day after treatment showed much greater differences between the treated and untreated animals.

TABLE 10.

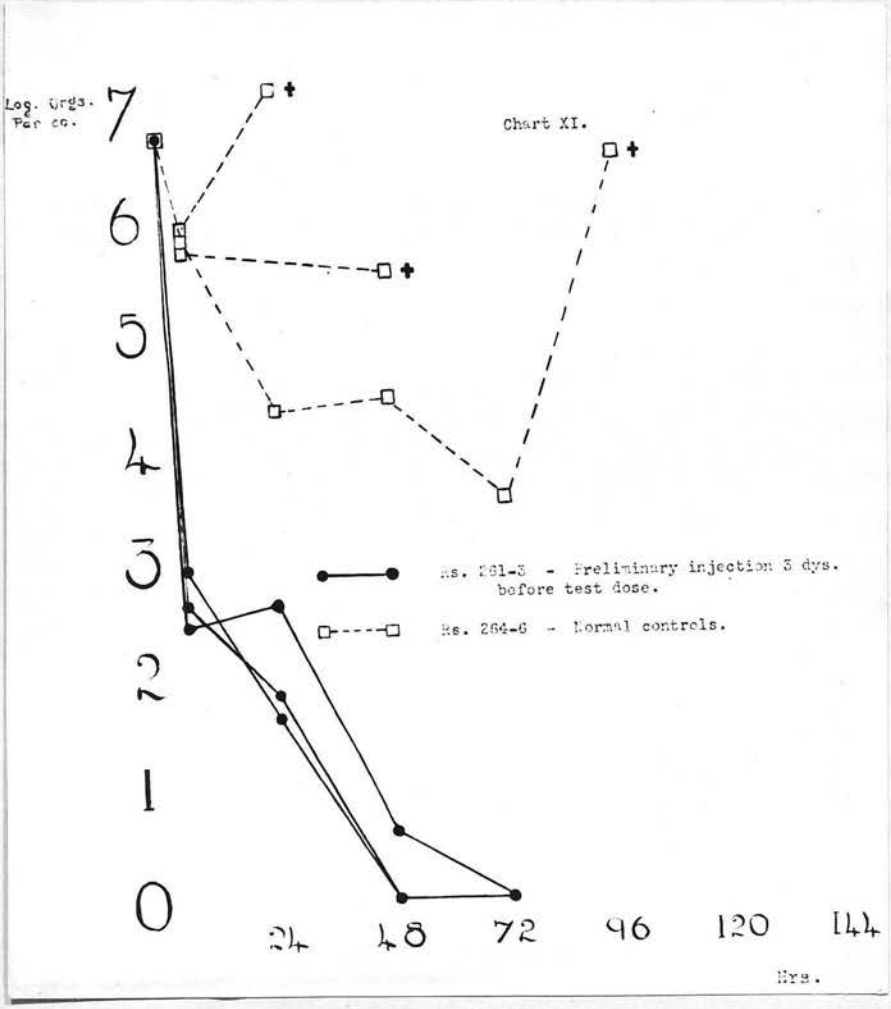
	Injection 3 days after inoculation of killed culture.					
	Treated.			Normal.		
Rabbit	261	262	263	264	265	266
Immediate	5,200,000	4,700,000	4,800,000	5,300,000	5,000,000	5,300,000
5 hrs.	822	244	356	701,000	807,000	459,000
24 "	36	424	62	21,656	13,500,000 (appr.)	?
48 "	0	4	0	25,600	D i e d.	330,000
72 "	0	0	0	3,000		Dead.
96 "	0	0	0	2,968,000		

Chart XI. shows these results in graphic form. (See p. 34). The effect of the immunising process upon the course of the subsequent bacteriaemia is now very apparent. The preliminary removal is very much more rapid and complete in the treated animals without exception and the subsequent period of increase much diminished or suppressed. The animal has at this date entered quite definitely upon that period of immunity which is characterized by marked increase in capacity to remove organisms from the circulation.

Experiments bearing upon the same point were made in which both the immunizing and the test dose consisted of living culture. For the former the amount used was 0.1 c.c. of culture; for the latter the usual amount of 1 c.c. It was in some cases quite impossible to interpret the results in detail in view of the fact that there was a bacteriaemia present at

the time the second inoculation was made. In many instances after the second day the second dose of organisms appeared to be completely removed at once and to have little, if any, effect upon the course of the bacteriaemia. In some cases the number of organisms in the blood five hours after the second dose was given was smaller than the number before the reinoculation.

CHART XI.



DURATION OF THE IMPROVEMENT IN CLEARING CAPACITY.

It was seen above (p.14) that one month after injection of a single dose of living culture the improvement in clearing capacity could still be demonstrated. Experiments on a more extended scale have been employed to determine how long such an improvement may exist. Six rabbits were given 5 c.c. of killed culture intravenously on 18-12-25. Two of these were given a test done on 18.1.26, two others a similar dose on 18.3.26 and the remainder on 5.7.26. That is, the capacity was tested after 1 month, 3 months and 6½ months respectively. The results are recorded in the following tables.

TABLE 11.

Injection One Month after treatment.			
	Treated.		Control.
Rabbit	297	298	305
Immediate	5,700,000	5,700,000	5,800,000
5 hrs.	2	8	20,000
24 "	13	0	53,000
48 "	0	0	2,500
72 "	0	0	12
96 "	0	0	0

TABLE 12.

Injection Three Months after treatment.				
	Treated		Control.	
Rabbit	299	300	247	248
Immediate	1,000,000	1,000,000	870,000	1,100,000
5 hrs.	12	68	1,300	3,300
24 "	0	289	142,000	1,953,000
48 "	149	79	2,800	Innumerable
96 "	0	0	D i e d	D i e d

TABLE 13.

Injection Six Months after treatment.				
	Treated.		Control.	
Rabbit	301	302	418	419
Immediate	3,600,000	3,900,000	5,400,000	5,400,000
2 hrs.	100,000	2,400	5,500,000	1,970,000
5 hrs.	10,700	206	Innumer-able	Innumer-able
24 hrs.	14,200	944	D e a d	D e a d
72 hrs.	3,026	106		
120 hrs.	0	0		

(In the last experiment the controls were much lighter than the test animals and the dose therefore higher. Accordingly a further experiment was made next day with a subculture from the

one used on this occasion. The animals used were slightly heavier than the test ones in this experiment. They both died in less than 24 hours as did the light controls).

In this series of experiments unfortunate variations in dosage and virulence render detailed comparison impossible but the three experiments taken together show that at the end of a month the improvement is still present practically undiminished; after three months it is also much in evidence but by the end of six months the course of the bacteriaemia is beginning to return to the normal type. There does appear however to be distinct evidence that some degree of immunity may last for as long as six months and the preliminary period of clearing is still a very active one.

In connection with the investigation of antibodies in the blood, experiments are recorded (p.60) indicating that a single dose of living organisms may produce an improved resistence and improved clearing capacity which are still in evidence 10 months later.

TRANSFERABILITY OF IMPROVED CLEARING CAPACITY.

The next step was to determine if the improvement in the rate of clearing could be transferred passively to another rabbit.

Rabbit 298 received 5 c.c. of killed culture on 18-12-25. One month later it was inoculated with 1 c.c. of living organisms. At the end of 5 days it was bled and its serum injected intravenously into two normal rabbits, 1 c.c. into rabbit 309 and 5 c.c. into rabbit 310. One hour later these two rabbits together with a normal control were inoculated with 1 c.c. of serum broth culture with the result recorded in the table.

TABLE 14.

Rabbit	309	310	311
Serum 298	1 c.c.	5 c.c.	Nil
	Organisms per c.c. of blood		
Immediate	2,600,000	2,300,000	2,300,000
5 hrs.	52	2	43,000
24 "	14	8	D e a d.
48 "	1	0	
96 "	0	0	

The serum in this case agglutinated in a dilution of 1 in 8 when tested at 55°C. for 2 hours. The actual

dilution in which it would be present in the rabbit would be about 1 in 20 in the case of 310 and 1 in 110 in 309.

It is clear that the injection of serum previous to inoculation of organisms does confer this capacity passively on other animals.

In another experiment the source of the serum (rabbit 290) had been treated with 5 c.c. of killed culture one month before the experiment. The serum did not agglutinate the homologous strain even in a dilution of 1 in 2 when heated for 2 hours at 55°C. A dose of 5 c.c. was injected intravenously into rabbit 306 and 1 c.c. into rabbit 307 and the test dose given on the following day to both rabbits and one control normal (308). In this case the rabbit which received 5 c.c. had removed the organisms from its blood in 5 hours, while in the other two cases they were found in considerable numbers (13,700 per c.c. in rabbit 307, 16,000 per c.c. in 308). There was however a slight return of the organisms on the following day. The effect was quite definite but was much less marked than in the previous experiment.

The serum of a normal rabbit administered 1 hour before a test dose has no such effect.

Further protocols are given on p.58.

APPEARANCE AND DURATION OF ANTIBODIES IN THE CIRCULATION AFTER IMMUNIZATION WITH KILLED CULTURE.

The phenomenon under discussion has been so far defined. It consists in the fact that a single intravenous injection of 5 c.c. of a killed culture of pneumococci confers on a rabbit a greatly enhanced capacity to remove a subsequent dose of living organisms from the circulation and suppresses, completely or in part the subsequent increase in numbers of these organisms. This effect, present to a slight extent within a few hours, becomes well established on the third day after inoculation; it is still well marked at the end of a month and may even be demonstrated after three or even six months, though, apparently in reduced amount. The power to behave in this manner may be transferred by means of the serum.

The improvement in the first few hours is slight and uncertain and apparently nonspecific. The really striking and apparently regular change occurs on the third day. An immunity phenomenon which lasts from 3 days to 6 months or more after an immunising injection suggests at first sight a cause which is different from the ordinary antibodies.

Agglutination Bull (1914 - 1916) has studied the phenomenon very thoroughly and at first attributed it entirely to the agglutinating action of the animal's blood.

The fact recorded above (p.38) that a serum can transfer this power in a dilution (1 in 110 in the rabbit) very much less than that (1 in 8) in which agglutination occurs in vitro, does not necessarily disprove Bull's theory. He suggests that this is because the reaction occurs better in vivo than in vitro and supports this view with some experimental evidence. It is possible that a similar explanation might apply to the other experiment on passive transference by means of serum in which no agglutinins could be demonstrated in vitro. The matter will be discussed further below. Here it will suffice to state that in response to a single dose of killed pneumococci agglutinins (as tested in vitro) are not always produced and, if they are, appear in small amount on the fourth day, increase to a maximum about the 7th to 10th day and disappear by the end of three weeks.

Complement fixing bodies have been studied but the difficulty in regard to them lies in the fact that they are present in considerable amount in normal rabbit serum. They are not greatly increased by this amount of immunization and appear to run fairly parallel to the agglutinins but the alteration is so slight that the results did not seem to be very reliable.

Opsonins. The serum of normal rabbits has been found to have no opsonizing effect upon the strain of pneumococcus used in these experiments. Four rabbits were given 5 c.c. of formalinized broth culture intravenously and the

opsonizing power of the serum tested before and at intervals after this treatment. No enhanced activity of the serum could be detected in any animal up to ten days after treatment. Blood prevented from clotting by the addition of 1 per cent heparin solution was inoculated with virulent culture and films examined after incubation of the mixture. In animals recently immunized with living culture (7 days before) following on preliminary treatment with killed organisms greatly enhanced phagocytosis has been observed; at later periods (one month and upwards) the power has not been in evidence. The development of demonstrable opsonins is, therefore, irregular and uncertain and does not parallel the development of active immunity.

Precipitins. These antibodies are developed with rather more consistency and may last much longer than agglutinins or opsonins, but in many cases they fail entirely to develop after a single dose of killed culture. If they do develop they usually disappear by the end of one month after treatment. An animal may develop a marked increase in clearing capacity without precipitins ever appearing in the serum and may continue to retain it long after they have disappeared.

EFFECT OF WHOLE BLOOD UPON PNEUMOCOCCI IN VITRO.

In view of the lack of correspondence between the occurrence of the ordinary antibodies and that of the improved capacity to deal with and remove organisms from the circulation, it seemed possible that some further information might be obtained from a consideration of the capacity of the whole blood to destroy or inhibit the multiplication of the organisms in vitro.

The first attempts were directed towards determining what number of organisms could be completely removed from the blood, several small (1 c.c.) samples being received into measured quantities of sodium citrate or heparin solution. Each of these was then inoculated with a measured volume of a dilution of culture, the series diminishing by multiples of ten. The tubes were then incubated at 37° C. for 24 hours at the end of which time they were examined culturally and microscopically to see whether growth had occurred or not. The number of organisms employed in each case was estimated from plate cultures made from a suitable dilution. This method suffers from two obvious disadvantages. In the first place the actual number of organisms introduced into each tube is not actually measured. It is merely estimated. In the second place it does not permit of the differentiation between growth inhibition and actual destruction. What seemed necessary was a method by which not only the end result of the

process but the intermediate stages might be studied. In order that this might be done the quantity of blood would need to be sufficiently large to permit of sampling at intervals.

The choice of the form which the "whole blood" was to take had to be considered. Heist & Solis Cohen (1919) and Heist, Solis Cohen & Solis Cohen (1918) used whole blood for similar experiments without attempting to prevent coagulation. This method would effectively prevent intermittent sampling and further it leaves the whole question unsolved because the factors of active bacterial destruction and the physical effects of coagulation are inseparably intermingled.

It was found that citrated blood gave unsatisfactory results. The main reason for this seemed to lie in two factors. In the first place the strength of sodium citrate required to prevent the clotting of rabbit's blood was in the neighbourhood of 0.3 to 0.4 per cent. There seemed to be quite considerable variations in different animals. On the other hand the growth of the pneumococcus was very definitely delayed by as little as 0.2 per cent of this solution and slightly inhibited even by 0.1 per cent in broth. There is thus a very small margin between the amount which is anticoagulant and what would be inhibitory to growth. Further the results obtained in experiments were quite irregular and quite disagreed with those obtained by other methods. In addition sodium citrate has been shown to interfere with antibody

reactions in a most powerful manner (Wright & MacCallum 1922) so tha even a small excess might very readily invalidate any results obtained.

Defibrinated blood has been recommended by Sir Almroth Wright and his co-workers as suitable for experiments of this kind. An objection to it is that during the process of defibrination the blood is considerably altered and deprived of fibrinogen and a considerable proportion of leucocytes. In actual practice I have found that the activity of defibrinated blood is distinctly less than that of the same animal's blood treated with heparin. This latter substance in a concentration of 0.2 per cent prevents clotting quite efficiently for 24 hours. Acting as it does upon the fibrin ferment it does not alter the more important constituents of the plasma (salts and proteins). There is however one point to be emphasized. If citrated blood be centrifuged at a moderate speed the plasma still contains the platelets which are quite discrete. If however heparinized blood be treated in the same way the plasma is quite clear, the platelets being deposited with the cells in relatively large clumps. To this extent at least the blood is not whole blood. I have satisfied myself that heparin has no inhibitory effect upon the growth of the pneumococcus in broth.

The technique finally employed was to place 1 c.c. of 1 per cent heparin solution in normal saline, sterilized by boiling, in a sterile tube and to bleed the rabbit from

the ear vein directly into this solution the tube being marked to indicate when 4 c.c. of blood have been added. The tube was vigorously shaken to ensure good mixing of the blood and heparin.

It was essential to maintain the blood at the correct temperature during manipulations. Incubation of the tubes within a bath of water in the incubator was tried with the idea that the large bulk of fluid would better retain its heat when outside the incubator. As the incubator was a dry one it was soon found that evaporation produced a considerable fall in temperature in the water bath as compared with that of the air in the incubator. It was found sufficient to incubate the tubes in the ordinary way and then transfer them rapidly from the incubator to a portable water bath at the appropriate temperature. While they were in this bath the necessary manipulations were carried out as quickly as possible, after which the cultures were removed from the bath and replaced in the incubator, the excess of water being removed from the outside. The method, though not ideal, seemed to be effective.

The culture to be used was diluted in saline containing gelatin and sodium phosphate according to the requirements of the experiment, a measured amount being then added to the tube of heparinized blood which had been incubated for a quarter of an hour to allow it to reach the appropriate temperature. A sample was taken immediately after inoculation and

plated. This was regarded as the inoculum. At selected intervals thereafter further samples were taken and similarly examined. Dilutions in the same diluting fluid were made where necessary.

GROWTH OF PNEUMOCOCCI IN BLOOD OF NORMAL RABBIT.

In this study attention was particularly directed to two main points:-

(1) the capacity of the blood to destroy organisms introduced into it:

(2) the inhibitory action of the blood upon the growth of organisms. In connection with this it seemed probable from the writer's previous work on streptococci (Wright 1925) and from earlier work upon the pneumococcus (Blake 1917) (Dochez & Avery 1916) that this would be found must marked in the early hours of growth. Accordingly it is the latent period which has been mainly investigated.

A considerable amount of work was wasted before it was realized how greatly the nature of the culture used affected the results. Chesney (1916) mentions that the precise form of the growth curve varies with the batch of medium employed. It seems that under certain conditions it also varies very considerably with the culture. The two cultures tested were both derived from one original culture - the same as used throughout these experiments. One had been maintained by subculture in broth and had become avirulent. The other had been maintained in a virulent condition in blood broth and when used was subcultured in serum broth. The following experiments show how these two factors, nature of

medium and virulence of culture, influence the length of the period of latency in the case of the pneumococcus. Tubes of broth and of serum broth (10% rabbit serum) in 5 c.c. quantities were inoculated with organisms from 24 hour cultures of virulent and avirulent pneumococci respectively and samples plated at hourly intervals.

TABLE 15.

Media	Organisms per c.c.			
	Virulent culture		Avirulent culture	
	Serum broth	Broth	Serum broth	Broth
Immediate	66	86	44	44
1 hr.	86	92	42	32
2 hrs.	86	86	38	20
3 "	112	94	78	28
4 "	296	56	132	18
5 "	908	72	448	36

In both cases the serum broth cultures seem to have begun to grow at 3 hours after inoculation whereas the broth cultures have not given clear evidence of increase at 5 hours. Other experiments have confirmed this and go to show that in in serum broth the latent period is about 2 - 3 hours while ordinary broth it is between 4 and 5 hours. One of the beneficial effects of serum on growth of the pneumococcus is

evidenced in this tendency for growth to begin earlier, a fact which has, so far as I know, no adequate explanation. I have failed to find any significant difference in this respect between virulent and avirulent cultures.

The results are however quite different if the experiment be made in the blood of a normal rabbit. Three rabbits were bled from the ear vein in the ordinary way into tubes containing heparin solution (14 c.c., 12 c.c. and 16 c.c. respectively into 4 c.c. of 1% heparin.) Each specimen was divided into two equal parts one of which was inoculated with 0.1 c.c. of 1 in 40,000 dilution of broth culture (avirulent) and the other with the same amount of serum broth culture (virulent), the cultures in each case being 24 hours old. Specimens were drawn at intervals and plated out, with the result recorded below. Two tubes of serum broth were inoculated as controls.

TABLE 16

Culture	Organisms per c.c.							
	Virulent.				Avirulent.			
	*1	2	3	Serum broth.	1	2	3	Serum broth.
Immediate	512	461	506	530	165	221	220	209
1 hr.	440	452	530	317	132	160	141	184
2 hrs.	450	580	528	418	90	153	52	200
3 "	770	806	653	600	60	91	55	274
4 "	1,140	1,500	1,320	905	60	64	37	687
6 "	4,300	5,700	6,200	5,013	14	8	5	4,210
8 "	29,000	38,600	43,000	30,300	14	2	10	29,000
10 "	195,000	201,000	327,000	177,000	30	0	40	153,000

* Refers to number of rabbit whose blood was used.

The behaviour of the two cultures was very similar in serum broth - the avirulent organism beginning to increase slightly later though the difference is more apparent than real. In all the blood specimens the virulent culture had begun to increase in 3 hours after inoculation, number 2 possibly slightly earlier than the others. The avirulent culture however in the blood of the same animals showed, in two cases, no sign of increase even after 10 hours, in the third there is a doubtful rise in the number of colonies after 8 hours' incubation. Further than that there is considerable reduction in the numbers of organisms inoculated. There would appear to be no good evidence of any power on the part of the normal rabbit's blood

to destroy virulent pneumococci whereas an avirulent strain may be considerably diminished in numbers or its latent period considerably prolonged. It is obvious that observations of this kind should be confined to organisms of the same degree of virulence as those used in direct experiment on the animal.

(NOTE. In cases where the avirulent culture has finally grown in rabbit's blood it has been found that the organism has been uncapsulated whereas the virulent has always been capsulated.)

GROWTH OF VIRULENT PNEUMOCOCCI IN IMMUNE RABBIT'S BLOOD.

The effect of the blood of immunised rabbits on the virulent strain was tested by the same method. The animals used had been immunized with a single dose (5 c.c.) of killed culture 13 days before. The blood was inoculated with 0.1 c.c. of a 1 in 40,000 dilution of serum broth culture.

TABLE 17.

	Organisms per c.c.	
Immune Rabbit	No. 341	No. 342.
Immediate	285	264
2 hrs.	75	125
4 "	6	21
6 "	6	4
8 "	0	0
24 "	0	0

Clearly the blood has been able to destroy this number of organisms entirely, the greater number disappearing in the first two hours. The next experiment showed that this destructive action is dependent on the presence of cells in the fluid. Two rabbits recently immunized were bled into heparin and the blood mixed*. Part of this was used as such (Whole Blood). The remainder was centrifuged and the plasma

* This was done as a large amount of blood was required.

removed. A further specimen was obtained from each and defibrinated by shaking with glass beads and the specimens pooled. This was centrifuged and the serum taken off. Each of these specimens was inoculated with 0. 1 c.c. of 1 in 40,000 dilution of serum broth culture and the course of growth followed.

TABLE 18.

	No. of organisms per c.c.		
	Blood.	Plasma.	Serum.
Immediate	358	375	344
2 hrs.	122	422	320
4 "	22	362	310
6 "	4	1,050	2,075
24 "	0	Film ++	Film ++

Neither the serum nor the plasma agglutinated the organism in 2 hours at 55°C. but the growth which appeared at 48 hours was distinctly floccular. The organisms were well capsulated.

The whole blood killed the organisms but neither the plasma nor the serum had any such effect. It would seem clear that the delay in beginning growth noticed in this as in previous experiments is an effect of the fluids but destruction of organisms is not produced by the fluids alone, though it is in the whole blood.

Complete sterilization of the blood is not observed at all stages of immunization and it would appear that there is considerable difficulty in comparing this killing power of the blood of the same animal at different times. Chief among the difficulties is the fact that the reduction in numbers appears to vary considerably according to the size of the inoculum. Four specimens of blood from rabbit 346 (immunized 6 weeks before) were inoculated with varying amounts of a virulent culture and observed in the usual way with the following results:-

TABLE 19.

	Organisms per c.c.			
	1	2	3	4
Immediate	1,500	100	28	7
2 hrs.	1,170	85	31	10
4 "	630	60	18	6
6 "	470	40	15	8

The answer obviously varies with the inoculum. The phenomenon here described is similar to that observed by Wright, Colebrook & Storer (1923) who interpret it as due to an epiphylactic response, the greater number of organisms stimulating the cells to a greater production of active substances . I think it might equally well be attributed to a phenomenon such as phagocytosis or agglutination in which chance of incidence with some other particle or cell plays an

important role.

In this particular experiment the organisms actually grew in the blood and attained large numbers. As the period after the immunizing dose becomes longer the actual diminution in the number of organisms becomes less marked and may disappear. But for several months this effect of lengthening the latent period remains recognizable.

In the following experiments a group of five rabbits which had received a single dose of 5 c.c. of killed culture one month before were examined in some detail to see what correspondence there was between the various antibodies discussed and their capacity to remove organisms from the blood after intravenous inoculation. The results obtained were as follows:-

TABLE 20.

Rabbits immunized one month before.

Antibodies in serum.					
Rabbit	377	385	356	394	462
Agglutinins	-	-	-	-	-
Phagocytosis (whole blood)	-	-	-	-	-
Precipitins	-	+	-	-	+

TABLE 21.

Growth of organisms in whole blood (in vitro)

R. No.	No. of organisms per c.c. 377	385	356	394	462
Immediate	240	228	170	216	182
2 hrs.	234	240	138	124	112
3 "	192	196	56	92	90
4 "	276	192	52	58	72
6 "	430	190	6	38	84

At 24 hours marked growth in all cases.

In all cases there is delay in the appearance of growth but the blood of rabbit 377 is less effective than the others. Two of the specimens show considerable diminution in the number of organisms during this period. In only two cases are precipitins observed. To test the protective action of the serum 5 c.c. from each rabbit were inoculated intravenously into a normal animal. At the same time 5 c.c. of normal rabbit serum were injected as a control into each of two normal rabbits. One hour later these rabbits and an untreated control normal were each inoculated with a test dose of culture and the blood was examined with the following results:-

TABLE 22.

Protective action of serum.								
Rabbit	480	481	482	483	484	485	486	488
Serum from	377	385	394	N.	N.	356	462	-
	Organisms per c.c. of blood in hundreds.							
Immediate	26,000	26,000	28,000	26,000	22,600	28,000	28,000	25,000
5 hrs.	259	170	88	2,900	900	11	6	820
24 "	128	99	238	Uncountable	3,660	170	14	28,500
	L.	S.	D. 5 days	D.48	D.72	L.	S.	D.72

D.= died. L.= developed local lesion. S.= survived.
N.= normal rabbit.

All the animals which received serum from a treated rabbit showed more complete removal of the organisms in five hours and less increase at 24 hours than those which received normal rabbit serum or the control. The sera containing precipitins (385 and 462) were more effective in preventing lesions and death but not in both cases in producing increased preliminary clearing. Without pressing the result too far there is some protective action present even after precipitins disappear and there is also growth inhibitory power present in the blood. The actual capacity of the animals from which the serum was derived to remove organisms was now tested in the usual way. The result is recorded in the following table, together with the results of the other tests and the results in two control normal rabbits.

TABLE 23.

Rabbit	377	385	356.	394	462	478	479
			No. of organisms per c.c.				
Immediate	1,030,000	1,170,000	1,170,000	1,250,000	840,000	950,000	1,070,000
2 hrs.	20,800	0	0	0	0	210,000	137,000
5 "	340	0	0	0	0	25,000	25,000
24 "	1,300	0	0	0	116	1,400,000	1,510,000
48 "	134	0	0	0	0	Dead	Dead
96 "	0						
Precipitins	-	+	-	-	+		
Growth delay	+	++	++	++	++		
Protection	+	++	+	+ (slight)	++		

The correspondence between active immunity and these other phenomena is not exact but it seems to be better with the capacity to delay growth than with the protection or precipitin tests.

Finally, a similar experiment, with the exception of the protection experiment, was carried out on a group of rabbits which had had one or two doses of killed or living culture or both at varying dates preceding the experiment. Their sera were tested for agglutinins and precipitins and the growth of the organism in heparinized whole blood was also examined. Finally they were all submitted to an inoculation of living

virulent culture intravenously to test their clearing capacity. In addition there were included two untreated rabbits. The blood of one of these (489) had shown a certain amount of delaying effect on the growth of virulent pneumococci and it was thought desirable to test its clearing capacity as well. This is the only untreated rabbit so far examined which has shown any sign of the delaying action of the blood upon growth. The results are given in the following tables:-

TABLE 24.

Growth of culture in blood (in vitro)

No. of organisms per c.c.

R. No.	273	292	302	313	342	362	489	Normal 492
Immediate	12,700	12,000	13,000	9,800	10,600	10,100	11,800	10,900
2 hrs.	8,300	9,700	8,400	9,200	7,000	8,500	6,500	12,000
3 "	22,100	9,100	5,800	10,100	7,800	7,500	5,400	20,900
4 "	77,000	10,500	5,200	8,200	13,500	19,500	5,600	68,000
6 "	553,000	22,000	2,900	28,900	73,000	107,000	32,800	567,000

TABLE 25.

Immediate*	Rate of removal of injected organisms from blood stream							
2 hrs.	23,200	30	0	0	40	71,800	18,400	340,000
5 "	250	0	0	0	0	1,960	170	58,300
24 "	216	44	0	8	218	1,600	354	120,000
72 "	0	0	0	0	0	20	1	24,000 Died.

* the dose was so adjusted for each animal as to give a total inoculation of 4 million organisms per c.c.

All the specimens show delay in growth of the culture inoculated into blood excepting the normal control and 273. Similarly all show a marked increase in clearing capacity including 273. Now the serum of these animals had been examined for agglutinins and precipitins two months before and none had been present except precipitins in the case of No. 302. From this serum they had also disappeared by the time of this experiment. The immunizing process had consisted of the following treatment:-

273	5 c.c. killed culture and 1 c.c. living	11 months before
292	1 c.c. living culture	10 months "
302	5 c.c. killed 10 months before and 1 c.c. living	3 months "
342	1 c.c. living	7 months "
362	5 c.c. killed	4 months "
313	1 c.c. living	8 months "
489) 492)	None	

The duration of the immunity after so little treatment is surprisingly long. It is not considered justifiable to do more than draw attention to the fact that these animals might have been considered to be devoid of antibodies but there is clear evidence of the presence of ENHANCED ACTIVITY OF THE BLOOD in all but one case if the growth experiment is carried out. Further the untreated rabbit whose blood had this capacity behaved like a slightly immunized animal The relation between delaying power of the blood and resistence is not quantitative and in the case of 273 antibody effects cannot be detected even by this delicate test.

The capacity to delay growth clearly long outlasts the presence of ordinary antibodies. It has also been shown that it may precede them in its appearance. Four rabbits used to test the course of opsinins following injection of killed culture were also examined to determine the presence of other antibodies in the serum and of growth inhibitory properties in the blood. In none of them did agglutinins, precipitins or opsonins appear up till the tenth day. By the third day two showed quite definite evidence of increased inhibition of growth in blood and by the fourth day the same power was manifested by the blood of all four rabbits.

DESTRUCTIVE POWER OF BLOOD COMPARED WITH PRELIMINARY CLEARING CAPACITY OF ANIMAL FROM WHICH IT WAS OBTAINED.

The capacity of the blood to destroy pneumococci in vitro is certainly increased by the process of immunization but it is clear that this capacity is by no means sufficient to account for the diminution which takes place in the blood in vivo either in the normal or the immune animal. This will be seen from the following examples.

TABLE 26.

Normal rabbits.

Organisms per c.c. of blood.				
Rabbit	347		348	
	In vitro	In vivo	In vitro	In vivo.
Immediate	506	870,000	461	1,000,000
1 hr.	530		452	
2 "	528		580	
3 "	653		806	
4 "	1,320		1,500	
6 "		1,300		3,300

In this case although the blood of the animal is practially devoid of power to reduce the small number of organisms inoculated into it, yet the animal as a whole has

a very remarkable ability to remove them from the circulation.

TABLE 27.

Immunized rabbits.

Organisms per c.c. of blood.				
Rabbit	385		394	
	In vitro	In vivo	In vitro	In vivo
Immediate	228	1,170,000	216	840,000
5 hrs.	190	0	38	0

The reduction produced in vitro is in neither case of anything like the same order as that in a corresponding time in vivo. In this experiment the amount of destruction or reduction that occurs in vitro is very slight and the inoculum small but even with larger inocula and much more highly active blood the number removed is never very great (several thousand per c.c.) and does not approach that which occurs within the animal when the organisms are introduced into the circulation.

It remains therefore to seek for some explanation of this discrepancy.

THE EFFECT OF TEMPERATURE UPON THE DESTRUCTION OF ORGANISMS BY BLOOD IN VITRO.

The experiments up to this point had all been made at a temperature of 37°C. Now the normal temperature of rabbits is definitely higher than this though it certainly varies considerably. I have selected the temperature of 39°C. as representing a fair average (= 102.2°F.) Further, following an inoculation of organisms there is frequently a considerable rise of temperature which in the course of 4 or 5 hours may reach 40°C. (104°F.) or 40.5°C. (105°F.)

Now the maximum temperature at which growth of pneumococci will occur is given by Muir & Ritchie as 42° C., by Hiss & Zinsser (quoting Fraenkel) as 41°C. The optimum growth occurs at 37°C. Possibly the destructive action of the blood might vary with the temperature.

A preliminary experiment showed that the effect of the temperature varied with the medium employed. Two tubes of broth, two of serum broth and two of normal rabbit's blood were inoculated with a measured amount of virulent culture of pneumococcus. One of each pair was incubated at 37°C. and the other at about 40°C. The course of events is recorded below:-

TABLE 28.

	Broth.		Serum broth.		Blood.	
	37°C.	40°C.	37°C.	40°C.	37°C.	40°C.
Immediate	28,000	23,600	27,000	24,100	24,900	22,300
2 hrs.	33,000	7,100	50,000	8,400	60,000	19,600
4 "	36,000	396	741,000	4,700	792,000	61,600
6 "	54,000	24	8,050,000	8,700	8,100,000	575,000
8 "	240,000	2	97,800,000	40,900	52,700,000	5,980,000
10 "	1,420,000	0	465,000,000	96,500	Too many to count.	16,380,000
24 "	Film +++	0	+++	10	+++	++

At the higher temperature the organisms disappear entirely from broth, diminish and then multiply slightly in serum broth, whereas in rabbit's blood good growth occurs, preceded it is true by a slightly longer latent period than occurs in the tube at 37°C. Extension of these experiments indicated that the results obtained varied very much from day to day and that slight variations in temperature made rather large differences in results round about 40°C. Accordingly accurately standardized thermometers were employed for the following experiments which were directed to determining whether there was any significant alteration in the destructive power of the blood at the higher temperatures.

TABLE 29.

Normal rabbits.

Growth in blood in vitro.

Number of organisms per c.c.

	R.478			R.479		
	37°C.	39°C.	40.5°C.	37°C.	39°C.	40.5°C.
Immediate	10,300	9,900	9,800	13,400	9,300	9,700
2 hrs.	11,900	9,400	7,000	16,800	9,900	6,400
3 "	34,000	11,600	4,800	20,500	16,800	6,400
4 "	60,000	25,100	2,300	109,000	41,000	4,900
6 "	749,000	228,000	4,500	947,000	262,000	10,000

In this case the blood was drawn from two normal rabbits and inoculated with organisms in the manner previously employed. There is practically no evidence of any destruction of organisms at either 37°C. or 39°C.:at 40.5°C. there is what I take to be a significant diminution in the numbers but small compared to the numbers removed from the blood in a corresponding period after intravenous inoculation which may be seen from the following table:-

TABLE 30.

	478	479
Immediately after inoc.	950,000	1,070,000
2 hrs.	210,000	137,000
5 "	25,000	25,000

What does appear however is that at the higher temperatures the commencement of active growth is delayed; slightly and uncertainly at 39°C., quite markedly at 40.5°C.

The blood of two slightly immunized rabbits was also examined in the same way; they had been inoculated 1 month before the experiment with 5 c.c. of killed culture. The figures obtained were:-

TABLE 31.

Growth in blood of immunized rabbits (in vitro) Organisms per c.c.						
	Rabbit 1.			Rabbit 2.		
	37°C.	39°C.	40.5 C.	37°C.	39°C.	40.5 C.
Immediate	106,000	89,000	91,000	101,000	117,000	109,000
2 hrs.	68,000	33,000	29,000	40,600	28,800	34,600
5 "	120,000	13,400	6,800	34,500	14,300	8,700

Here there would appear to be rather more destruction of organisms at the higher temperature but the actual amount in no way compares to what happened in the animal. The same animals were inoculated with 1 c.c. of culture and were able to reduce the numbers in 5 hours from 1 million per c.c. to 0 in the case of rabbit 2 and to 340 in the case of rabbit 1.

The effect of the higher temperatures would seem to be not so much in increasing the activity of destructive agents that may happen to be present in the blood, the difference not

being very marked in the first 2 hours, as in lengthening the period during which they have an opportunity of unhampered action owing to the fact that the organism is not multiplying.

The conclusion seems justified that, whatever be the value of the study of the blood in this way as an index of immunity or resistence, it is by no means a complete indicator of the total capacity of the animal.

PHASE OF GROWTH OF THE ORGANISM AT VARIOUS STAGES OF THE SEPTICAEMIA.

In the earlier experiments it was shown that during the first two hours after intravenous inoculation of organisms from a 24-hour culture, reduction in numbers goes on at a very rapid rate. The reduction may go on at a slower rate until five hours from the injection when either a definite rise occurred or the numbers remained fairly steady. Later it was found that in some animals the organisms commenced to increase in numbers at the end of two hours. I have never observed a case in which this increase has begun before that time. The foregoing experiments upon the growth of virulent organisms in vitro in rabbit's blood had shown that for a period of two hours after introduction the 24-hour serum broth culture fails to grow, that is, fails to increase in numbers as judged by colony counts. The culture therefore was in the phase known as the period of latency. It is quite possible that the organism at the time of the increase of numbers within the animal was simply beginning to multiply after the ordinary period of inactivity. This was tested in the following experiment.

Two rabbits (420 and 421) were each inoculated intravenously with 1 c.c. of culture. At the time of inoculation a measured amount of the culture was inoculated into serum broth and the course of its growth examined. The latent period

was found to be about 2 hours. One hour after the intravenous injection blood was drawn from the ear as quickly as possible into heparin at a temperature of 39°C. and the tubes immediately placed in a bath at the same temperature. Specimens were plated at once and at intervals of 1 hour thereafter. Similar specimens were prepared at 5 hours and examined in the same way. The following are the figures obtained:-

TABLE 32.

Organisms per c.c. Specimen of blood obtained 1 hour after inoculation.		
	R.420	R.421
Immediate	1,040,000	960,000
1 hr.	940,000	1,090,000
2 hrs.	1,810,000	2,260,000
3 "	4,490,000	5,250,000

During the first hour in the test tube no multiplication occurs, but during the next hour there is a very marked increase in numbers. That is, the organisms in the blood of the animal one hour after an intravenous injection are still in the latent phase and remain so for one hour instead of the original two hours.

TABLE 33.

Specimen obtained 5 hours after inoculation.

Number of organisms per c.c.		
	Rabbit 420.	Rabbit 421.
Immediate	510,000	1,290,000
1 hour	1,700,000	4,390,000
2 hrs.	3,440,000	9,500,000
3 "	10,100,000	21,300,000

In this specimen it is evident that during the first hour in the test tube a great deal of multiplication has taken place and that the organism was, at the time when this sample was taken, in the phase of active growth. All experiments directed to this point have given the same result.

A further experiment was done upon another animal, the blood being drawn upon the 3rd day after inoculation (the animal died on the fifth day). The temperature of the rabbit at the time the blood was taken was 39.6°C. Incubation of the sample was at 39.2°C. and the results were as given.

TABLE 34.

Specimen of blood on 3rd day after injection.

Number of organisms per c.c.	
Immediate	68,700
1 hour	276,000
2 hrs.	476,000
3 "	1,076,000

Here too there was obviously no delay before multiplication occurred and the organism in the rabbit's blood at this time was in the phase of active growth.

In these points the behaviour of the organism within the animal does appear to be analogous to what occurs in the test tube. The ordinary 24 hours culture employed is in a period of latency at the time of inoculation. One hour later the organism is in the same phase but it is now of shorter duration - one hour. At the end of five hours the organism has no such latent period but is in the phase of active growth.

This change of phase would account quite well for the alteration from rapid reduction to relatively rapid increase which occurs in a certain number of animals. But the time relations are not the same in all cases. Is there any corresponding variation in the length of the period of latency? To test this a series of six normal rabbits of different weights were selected, samples of blood drawn in the usual way and the course of growth of an inoculum observed. By choosing animals of different weight it was hoped that the natural variations might be more readily observed. The following day the same rabbits were inoculated intravenously with a dose of culture, graded according to weight, and the course of the bacteriaemia followed by means of hourly specimens of blood.

TABLE 35.

Growth curve in blood of series of six rabbits at 39.4°C.

Number of organisms per c.c.						
Rabbit	452	453	454	455	396	397
Weight(grams)	1950	1950	1400	1420	1000	1050
Immediate	4,750	4,680	4,500	4,600	4,200	4,200
1 hr.	4,130	3,870	3,830	4,030	3,630	3,840
2 hrs.	4,300	3,800	4,170	3,890	3,800	3,500
3 "	5,430	5,600	6,500	5,400	5,400	5,100
4 "	15,400	11,400	25,100	17,500	12,100	13,800
5 "	32,200	26,500	52,000	50,700	37,400	34,200
6 "	93,000	116,000	218,000	184,000	182,000	109,000
7 "	323,000	254,000	695,000	586,000	477,000	342,000

TABLE 36.

Course of bacteriaemia following injection of 1 c.c. of culture.

Immediate	3,480,000	3,450,000	3,600,000	3,500,000	3,360,000	3,200,000
1 hr.	180,000	960,000	1,030,000	1,220,000	1,490,000	1,130,000
2 hrs.	17,100	500,000	340,000	790,000	650,000	630,000
3 "	3,500	120,000	360,000	1,000,000	410,000	500,000
4 "	300	130,000	D i e d	1,640,000	720,000	710,000
5 "	200	80,000		3,800,000	1,310,000	1,220,000
6 "	< 100	150,000		11,100,000	1,770,000	2,020,000
7 "	< 100	230,000		22,900,000	2,800,000	3,270,000

I have underlined the figures which seem to indicate the point at which growth had begun to occur in the test tube and increase in numbers in the animal.

These are summarised as follows:-

Period at which increase had begun.

	452	453	454	455	396	397
In vitro	3 hrs.	3 hrs.	3 hrs.	3 hrs.	3 hrs.	3 hrs.
In vivo	>7 hrs.*	6 hrs.§	? 3 hrs.	3 hrs.	4 hrs.	4 hrs.

* Increase marked in 24 hrs.
§ except for the figure at 5 hrs. which is a little anomalous - this might possibly be regarded as a stationary period from 3 - 6 hrs.

It is clear that in four cases there is a certain relationship between the figures but it is not a precise one. The case showing the greatest disparity between the two sets of figures is rabbit 452. It is to be observed that the rate of reduction of the organisms in this animal is very much higher and the level reached considerably lower in the three-hour period than in the other animals. In the test tube growth occurs and its progress is unhampered whereas in the animal the eliminating mechanism is functioning actively. The rate of increase in the test tube is in almost all cases much greater than it is in the animal It would appear that multiplication in the animal has some brake acting upon it. It is nett not gross - the balance between total increase and elimination.

If the rapid rate of removal in the preliminary period is due to the fact that the organisms are not at that time multiplying, the course of the curve of the septicaemia should be considerably altered if the organism used for inoculating the animal were in the phase of active growth. Three rabbits were therefore inoculated each with 1 c.c. of an actively growing (6 hour) serum broth culture of virulent pneumococci, care being taken at the time of inoculation to avoid any serious change in temperature in the culture from that of the incubator. Cultures were made from the ear vein at intervals of one hour with the following result:-

TABLE 37.

Course of septicaemia following injection of actively growing culture.

Rabbit	252	468	490
	Organisms per c.c. in millions.		
Immediate	8	8	8
1 hour	14.1	13.4	12.5
2 hours	21.7	27.2	23
3 "	29.7	41.3	46.7
4 "	54.6	71.8	94.9
5 "	79.5	94.6	121 6

The use of such a culture has the effect in these cases of eliminating altogether the preliminary period of decrease in numbers, the increase being evident at the end of one hour.

CHANGES IN TEMPERATURE AND THEIR EFFECT UPON THE COURSE OF THE BACTERIAEMIA.

Experiments on growth in vitro are necessarily carried out at a fixed temperature. In the animal body, on the other hand, the temperature undergoes considerable variation. I have tried to determine if the course of the temperature in the animal might account for the greater length of time which elapses before increase in numbers occurs in vivo as compared with the test tube experiment. While the experiment recorded in Table 35, p. 73, was in progress the course of the rectal temperature in the animals was observed. Table 38 gives the result of these observations.

TABLE 38.

Rectal temperature of normal rabbits after inoculation.

	452	453	454	455	396	397
Before inoc.	38.5	39	39.1	38.5	38.8	38.9
1 hr. after	38.9	38.9	39.1	38.2	39	38.9
2 hrs "	40	39.8	38.6	38.6	40.2	39.2
3 " "	40.5	40.3	39.3	38.9	39.7	39.8
4 " "	40.1	40.6	39.3	40	40.4	40.8
5 " "	40.4	40.6	Dead	39.9	39.9	40.8
6 " "	40.2	40.3		40	40.1	41
7 " "	38.2	39.6		39.9	39.9	40.8

During the first hour after inoculation the temperature is little altered. During the second hour a rise occurs varying in amount in rabbits 452, 453 and 396. In the remaining cases the rise is later. I have underlined in each case the first record of a temperature of 40° C.

It is clear from this experiment that the greater part of the removal of bacteria from the circulation is done at a time when the temperature of the animals inoculated is not greatly different from that of the blood used for the experiment on growth in vitro (39.4°C.). Accordingly the difference between the destructive action of blood in vitro, and the clearing capacity of the animal as a whole cannot be accounted for in this way.

If we compare the time of the first important rise of temperature with the time at which the increase in the numbers of organisms occurs in the blood of the same animal we get the following table.

Rabbit no.	452	453	454	455	396	397
Time of rise of Temperature	2 hrs.	2 hrs.	Never	4 hrs.	2 hrs.	4 hrs.
Time of bacterial increase.	>7 hrs.	? 6 hrs.	? 3 hrs.	3 hrs.	4 hrs.	4 hrs.

Clearing it is not possible to associate the two phenomena very closely.

The experiments upon the effect of temperature on growth in vitro showed that a temperature of 40.5°C. had a marked effect in delaying the commencement of growth. It is interesting in this connection to compare the results of observations upon temperature and bacterial growth in vivo in the case of rabbit 397.

TABLE 39.

	Temperature °C.	Bacteria per c.c. of blood.
At time of inoculation	38.9	3,200,000
1 hr.	38.9	1,130,000
2 hrs.	39.2	630,000
3 "	39.8	500,000
4 "	40.8	710,000
5 "	40.8	1,220,000
6 "	41	2,020,000
7 "	40.8	3,270,000

Here there is clear evidence that during the 4 - 7 hour period the temperature of the animal is at about 41°C., very close to the upper limit of growth of the organism as ordinarily described. Yet during the corresponding period there is ~~xxxxx~~ a definite increase in the number of organisms in the blood. Now it is probable that in this animal the organisms during the period of relatively low temperature had entered upon the period of active growth. The following experiment shows that the effect of temperature upon growth is greatly modified by the condition of growth in which the organism happens to be at the time when it is inoculated.

Two specimens of heparinized blood from the same normal rabbit were warmed to 40.5 °C. and then inoculated with

measured amounts of serum broth culture, in one case derived from a twenty four hour, in the other from a six hour culture the former being in the phase of latency, the latter in the phase of active growth. The observations upon the course of growth gave the following figures:-

TABLE 40.

Growth at 40.5°C. No. of organisms per c.c.		
	24 hr. culture.	6 hr. culture.
Immediate	3,590	13,400
2 hrs.	1,230	79,400
4 "	420	421,000
6 "	180	825,000
7 "	300	

The inoculum consisting of organisms in the phase of latency is kept in that phase for at least 6 hours in this case, whereas the inoculum which is in a condition of active growth at a temperature of 37°C. is able to go on growing at once at the higher temperature of 40.5°C.

These results being somewhat inconclusive as to the role of temperature in natural resistence it was desirable to see if rabbits with a high temperature at the time of inoculation showed any distinct differences in the mode of dealing with the inoculum as compared with animals of normal temperature. Three animals were inoculated intravenously with 1 c.c. of formalinized culture of B.typhosus, three others with 5 c.c.

of formalinized culture of pneumococcus and two others were untreated. The rectal temperature was observed hourly. All eight rabbits were then (5 hrs. later) inoculated with 1 c.c. of living culture of pneumococci. Rectal temperatures were recorded hourly thereafter and cultures made from the blood at 2 hours and 5 hours after inoculation with the living organism.

TABLE 41.

Inoculated with	Typhoid.			Pneumococci.			Nil.	
Rabbit No.	399	402	458	414	415	460	459	461
Temp. at time of inoculation.	40.6	40.7	40.1	40.5	40.7	41.3	38.7	38.3
Organisms at time of inoculation (thousands)	4,600	4,900	4,700	4,900	4,500	4,800	4,500	5,100
Temp. 1 hour	40.4	40.1	39.6	40.2	40.4	41.4	38.9	38.9
Temp. 2 hours	40.3	40	39.2	40.3	40.8	41.9	39.7	39.3
Organisms 2 hrs. (thousands)	490	1,170	180	1,120	640	1,080	630	960
Temp. 3 hours	40.4	40.4	39	40.2	40.9	41.8	40	40.3
Temp. 4 hours	40.3	40.7	40	40	40.9	41.7	40.1	40.7
Temp. 5 hours	40.2	40.6	40.6	39.7	40.6	41.6	40.6	40.7
Organisms 5 hours (thousands)	100	1,050	66	930	82	82	440	1,880

This result does not appear to show any very marked constant difference between the rabbits with an initial high temperature and those with a low one. Certainly none of the animals with an initial high temperature show any increase in numbers in the

2 - 5 hour period whereas one of the normals does do so. No final conclusions appear to me possible but it does seem clear that results obtained in experiments in vitro cannot be transferred without further experiment to the conditions as found in the animal.

Attention is drawn to the figures for rabbit 460 in which the temperature is approaching that of the normal pigeon. Reduction in numbers is less marked in 2 hours in this animal than in No. 458 whose temperature is nearly 2 degrees less. It would seem to support Kyes's view (1916) that in the case of the immunity of the pigeon to the pneumococcus something more than the temperature has to be considered though this may certainly play a part.

ROLE OF THE LEUCOCYTES IN THE REMOVAL OF BACTERIA FROM THE BLOOD.

The destructive functions of the blood drawn from the animal and examined in vitro are manifestly not sufficient to account for the phenomena of removal of bacteria observed in experiments upon the living animal. In the test tube the conditions are relatively static and extremely artificial; in the living animal they are liable to wide variations and there are certain factors which are not represented at all in the test tube experiments. This is particularly the case with regard to the cellular elements. The alterations which occur in the peripheral blood in normal rabbits in the period following an intravenous injection of 1 c.c. of culture of virulent pneumococcus will be seen in the following table.

TABLE 42.

Leucocyte counts in normal rabbits.

No. of leucocytes per c.mm.		
	R.355	R.371
Before inoculation	6,200	5,400
10 mins. after	4,900	3,100
30 " "	3,800	2,900
1 hour "	2,800	2,400
2 hrs. "	3,900	2,800
4 " "	3,400	3,400
6 " "	3,000	4,600
24 " "	2,500	5,600
48 " "	3,300	

There was a well marked reduction in the peripheral leucocytes in the course of 10 minutes followed by a gradual return to normal or a maintenance at the low level for some time. This phenomenon was shown by Andrewes(1910) to be due to redistribution of the cells within the animal, especially a concentration of them in the lungs. The succeeding leucocytosis that is usually described was not seen in these cases.

In animals that are lightly immunized the course of the leucocyte count has been found to be somewhat variable as the following figures show:-

TABLE 43.

Leucocyte counts in immunized rabbits.

Number of leucocytes per c. mm.		
	R.211	R.214.
Before inoculation	4,800	4,100
10 mins. after	1,500	1,100
30 " "	3,000	3,000
1 hour "	4,300	2,400
2 hours "	8,000	3,000
4 " "	12,400	4,000
6 " "	12,800	3,500
24 " "	4,800	4,700
48 " "	5,400	

The findings in the first rabbit (211) suggested the occurrence of a specific leucocytosis such as is discussed by McWilliams (1916), Zinsser & Tsen (1917), and Gay & Claypole (1914). But it was the only one of my small

series which showed this. To avoid the variations occurring in different rabbits observations were made in the same animal after a first injection of organisms and after a second injection a week later.

TABLE 44.

Leucocyte counts after 1st and 2nd injections.

Number of leucocytes per c. mm.		
	1st injection	2nd injection.
Count before inoculation	5,400	5,200
10 mins. after	3,100	1,400
30 " "	2,900	2,700
1 hour "	2,400	2,200
2 hours "	2,800	2,600
4 " "	3,400	3,400
6 " "	4,600	4,000

The only difference appears to be in the degree of the preliminary diminution. Apparently the factors which bring this about are more active in the case of the immunized animal than the normal. Andrewesobserved this difference and he also found the leucopenia to be longer maintained as immunization progressed. This greater tendency of the leucocytes to aggregate in the lungs, particularly, occurs just at the time when the maximum removal of organisms from the blood is also occurring. Anything in the nature of a leucocytosis is entirely lacking during the very active clearing process in the immune animal.

I have tried to test the importance of the role of the leucocytes by diminishing the numbers of circulating cells by means of subcutaneous injections of benzole. Three immune and three normal rabbits were given subcutaneous injections of 2 c.c. of a mixture of equal parts of benzole and sterile olive oil per kilo of body weight every day as recommended by Selling (1911). The action of the benzole was found to vary a good deal in different animals, the course of the leucocyte counts being somewhat irregular. Two animals, one normal and one immune, died before the number of leucocytes was sufficiently reduced. The other animals bore the rather drastic treatment very well as shown by the following observations on their weight.

Weight of animals tested with benzole (in grams.)				
Rabbit	327	310	343	338
Before treatment	1,620	2,150	1,700	1,490
At end of treatment	1,620	2,100	1,720	1,520

Apparently if benzole is persisted with until the cells are completely removed death occurs in a large percentage of animals. The experiments upon removal of bacteria were therefore made upon animals whose leucocytes had been reduced to about 1,000 per c.mm. or less. The animals, with an equal number of controls in as nearly as possible the same condition regarding immunity as the test animals, were inoculated with 1 c.c. of culture and the

course of the bacteriaemia recorded.

TABLE 45.

Removal of bacteria in normal and benzolized unimmunized rabbits.				
R. No.	343	338	340	336
Leucocytes per c. mm.	1,300	700	5,300	5,500
Bacteria per c.c. of blood (in thousands)				
Immediate	14,000	15,000	16,000	16,000
2 hrs.	2,331	2,142	2,898	2,142
5 "	1,179	1,327	Too many to count	1,764

TABLE 46.

Similar experiment in immunized rabbits.				
R. No.	310	323	327	326
Leucocytes per c. mm.	800	7,200	400	5,300
Bacteria per c.c. of blood.				
Immediate	16 million	17 million	10 million	8 million
2 hours	0	0	36	10
5 "	0	0	0	8
Leucocytes 24 hrs. later	1,300	9,500		

From this it appears that a very considerable reduction in the leucocytes may be made without seriously affecting

the capacity of the animal to clear its blood of bacteria in the numbers here employed.

EFFECT OF ATTEMPTS AT BLOCKADE OF THE RETICULO-ENDOTHELIAL SYSTEM.

One important factor not represented in the test tube experiments is the system of phagocytic cells lining certain portions of the circulatory system and forming part of what Aschoff has called the reticulo-endothelial system. It is thought by many that the activity of this system may be diminished if the animal is given injections of some substance which this system of cells is capable of absorbing - the so-called "blocking" or "blockade" of the system.

I have tried to see if such treatment had any marked effect upon the removal of bacteria from the blood stream.

Four rabbits, two immunized and two untreated, were given a series of injections of Indian ink. The preparation used was Watson's drawing ink which was selected because of its marked stability in salt solution, a dilution of 1 in 10 in 0.85% sodium chloride solution showing no sign of precipitation even after autoclaving and subsequent standing at room temperature for five days. In the doses used - 5 c.c. of a 1 in 10 dilution in salt solution - it has appeared to be quite non toxic for rabbits, provided the injection has not been given quickly. The particles of which it consists are very fine and extremely even in size. Preliminary observations showed that it was readily taken up by Kuppfer's cells in the liver, cells of the spleen pulp and bone marrow, and

after intraperitoneal injections by the cells of the taches laiteuses of the omentum and by the lining cells of the sinuses of the lymph glands.

The experiment, to have much chance of success, has to be carried out within a short time so that compensatory proliferation of the cells of the system may be avoided. It was found that a single injection of this preparation gave a good demonstration of the system. Three such intravenous injections and one intraperitoneal were given to each animal within a week Two days after the last intravenous injection the rabbits thus treated, together with four control animals - two normal and two immunized - were given 1 c.c. of culture intravenously and observations made upon the septicaemia.

TABLE 47.

Effect of injection of Indian ink upon clearing power.

Unimmunized rabbits.				
	Treated with ink.		Untreated.	
R. No.	320	321	322	323
	Organisms per c.c. of blood.			
Immediate	1,900,000	2,500,000	2,200,000	2,200,000
5 hrs.	16,300	4,100	144,000	4,100
24 "	877,000	123,000	301,000	580

TABLE 48.

Immunized rabbits. (Last dose 3 weeks before).

	Treated with ink.		Untreated.	
R. No.	296	297	310	298
	Organisms per c.c.			
Immediate	1,700,000	2,000,000	1,700,000	2,200,000
5 hours	0	0	0	0
24 "	8	0	0	6

The experiment with the normal rabbits, while somewhat unsatisfactory owing to the wide divergence in the observed numbers in individual animals, does not show any definite difference between the two sets of animals in the matter of preliminary clearing. There does seem to be a slight tendency for greater increase to occur in the next 24 hours in the treated animals. The immunized animals showed no significant difference in either respect. Similar experiments were next made with injections of saturated solution of lithium carmine (Grubler) given intravenously in doses of 5 c.c. on four consecutive days. Twenty four hours after the last injection the culture was injected intravenously.

TABLE 49.

Effect of injection of lithium carmine.

Unimmunized rabbits.				
	Treated		Untreated.	
R. No.	331	333	375	376
	Organisms per c.c.			
Immediate	9,000,000	6,400,000	7,300,000	7,000,000
2 hrs.	2,020,000	430,000	90,000	420,000
5 "	1,950,000	1,700	860	34,600
24 "	D e a d	1,200	17,000	51,000

This experiment again suffers from the defect that these normal animals varied so considerably in their power of removing the organisms. The treated animals have however not lost the power to remove pneumococci. To test the matter further two rabbits were chosen which had been treated with living pneumococci4 months and 5 months before respectively. These rabbits being in a relatively weak state of immunization would be expected to show a considerable alteration in power if blocking the system removed their main eliminating mechanism.

TABLE 50.

Effect of lithium carmine injections.

Immunized rabbits.			
	Treated.		Untreated.
R. No.	290 (4 mos.)	291 (5 mos.)	314 (4 mos.)
	Organisms per c.c.		
Immediate	1,300,000	1,400,000	1,350,000
2 hours	0	4,300	2
5 "	0	102	0
24 "	0	1,352	32

Rabbit 290 removes the organisms just as readily as rabbit 314 which received no carmine. In the case of rabbit 291 the power is not so marked but the process does not differ markedly from what has been found quite commonly in animals at this period after immunization.

These experiments seem to show that the amount of treatment described did not seriously interfere with the animal's capacity to dispose of the injected organisms. Certainly the results in unimmunized animals were irregular and too much should not be deduced from them but where conditions were much more standardized, as in the immunized animals, it was not possible to find any significant difference between those which received injections of ink or carmine and those which did not.

ROLE OF THE BLOOD PLATELETS.

Various workers Delrez & Govaerts,(1918), Teale & Bach (1920), had suggested that the platelets played a predominant part in the removal of bacteria. Govaerts (1921) and Bull & McKee (1922) have however quite conclusively shown that the elimination of the platelets by means of an anti-platelet serum has no effect upon this activity. Bull's experiments were done in part upon the pneumococcus and it has not seemed necessary to go into the matter any further.

Here then we have three factors, each of which can be greatly modified independently without seriously prejudicing the animal's powers - the leucocytes, the reticulo-endothelial system and the blood platelets. Does this mean that none of these play any part or is it that the phenomenon is really a very complex one which involves all these factors and perhaps others? The experiments which have been described all tend to show that the immunized animal differs only quantitatively from the unimmunized. There is apparently an improvement in the capacity to dispose of organisms, fairly abrupt in its onset and gradual in its disappearance. Phenomena observed in the immune animal are probably therefore only an exaggeration of what occurs in the normal. With this idea observations were made to see what factors did actually take part in the removal of pneumococci from the circulating blood of an immune animal.

HISTOLOGICAL OBSERVATIONS.

The only two points to which I have devoted attention are (1) the factors concerned in the removal of organisms in the immunized animal; (2) the chief points of difference between the normal and the immunized rabbit.

It seemed advisable to make these observations early in the period of elimination as at this time the phenomena might be expected to be at their height and destruction of the bacteria would not have assumed large enough proportions to interfere with the results. Animals previously immunized with at most two doses of pneumococci, the last dose having been given a week before, were injected with 20 c.c. of virulent pneumococci. They were killed by inhalation of chloroform after 10 - 15 minutes and preparations made from peripheral blood, blood from liver, lungs and spleen xxxx and pieces taken for sections from the same organs. In some cases blood films from the ear vein were made at short intervals during the time between inoculation and the death of the animal.

The method of making films from the blood in the organs has appeared to be of importance. If the blood is obtained by puncture of the surface of the organ and collected as it escapes the films are practically devoid of leucocytes and, in the immunized animals, of organisms as well. In the normal animals there are also few white cells but the cocci are very numerous. To obtain films containing cells it is necessary to allow the organ to bleed freely after

incision and to rub the cut surface upon the slide. It seems as though the free bleeding of the organ, the blood presumably coming from the larger vessels, does not lead to the removal of either cocci or leucocytes in the immune animal. These are apparently present only in the smaller capillaries and require some force to dislodge them. The films prepared in this way were stained with Leishman's stain.

The preparations from the spleen did not give very much information in any of the cases examined and it would appear that little activity is to be expected in this organ at this stage (10 mins. after inoculation).

The preparations from the lungs of the recently immunized rabbits showed a most extraordinary phagocytic activity on the part of the leucocytes. Both polymorphonuclears and monocytes were engaged in this activity and in about the same degree. Practically all the bacteria seen had undergone some alteration in distribution; that is to say there were practically no isolated pairs of pneumococci. The changes consisted in

(1) phagocytosis by polymorphonuclears and monocytes;

(2) aggregation into masses;

(3) inclusion of clumps of organisms in masses of platelets.

In one particularly good preparation a count was made with the following result:-

Total number of organisms seen	893
Phagocyted by polymorphonuclears	297
" " monocytes	340
Total phagocyted	637
Total outside phagocytes	256
In unphagocyted aggregates associated with platelets	203
In free aggregates	53

It is not to be imagined that this degree of activity is to be seen in all preparations but it serves to indicate how quickly phagocytosis occurs and how extensive it may be.

The appearances seen in films from the liver are very similar but here the counts suggested that mononuclear cells were more active. This was because there were included four large cells of an obviously different type which contained between them/185 pairs of pneumococci; one cell alone contained 82 pairs. These were, I think, detached Kupffer's cells.

The sections of the organs, though less beautiful demonstrations confirmed these observations. In the spleen there was little phagocytosis and organisms were much fewer than in the liver and lung. In the latter masses of cocci were to be seen both within the cells in the capillaries and free in these vessels. Hardly any organisms were seen in the larger vessels. I could not satisfy myself that there was any phagocytosis by the capillary endothelium. In the liver

the most striking feature was aggregation of the organisms in the sinuses close to the wall and for the most part apparently phagocyted by Kupffer's cells. It was often difficult to decide if a given mass was within a cell or not especially if the nucleus did not happen to be in the section.

In less highly immunized (up to one month after treatment) animals these appearances are not so clearly seen. As the time since the immunizing dose increases the evidence of phagocytosis becomes less. One can however usually find some evidence of it. And at the same time there seems less tendency to form aggregates.

In the normal animal at this stage I have not observed this phagocytosis or marked tendency to aggregate that is seen in the immune. This does not mean, I think, that such phenomena do not occur. The dose employed here is an enormous one. It needs to be in order that the organisms may be seen. With the organisms are injected large amounts of the aggressin like specific soluble substances whose deleterious action on the activity of the leucocytes has been described by Sia (1926). Consequently a good deal of the activity of a normal animal towards a small dose of organisms might well be paralysed by the large doses here employed. It is possible that better results might be obtained at a later period.

In the films from the peripheral blood of immunized animals the phenomena of clumping of organisms and aggregation in masses of platelets were both observed. Phagocytosis was

not commonly seen. I have however seen one or two polymorphonuclear cells containing a few pairs of cocci.

Whether the platelet aggregations which occur in these films actually occur in the body of the animal I am unable to say. In my films about 50 per cent of the aggregates of platelets contained pneumococci while the others did not.

These observations then show that the removal of bacteria from the circulation in the immunized animals is due to their concentration within the capillaries of the viscera particularly the liver and lungs, partly in free aggregates, partly phagocytosed. In this process of phagocytosis the cells of the blood and those lining the sinuses of the liver are both concerned. Even with these enormous doses a very considerable portion of the cells are still disengaged, showing that there is a considerable reserve of cells available even without the intervention of any enhanced production by the blood forming organs. Further, both the granular cells and thecells of the reticulo-endothelial system take part. With so small a dose as the test dose employed in the experiments (1 c.c. of culture) the reserve available must be very great indeed. Laming of one mechanism would leave the other uninvolved. It is not surprising therefore that the experiments with benzole and with Indian ink and carmine failed to produce any noticeable effect on the clearing process.

AGGREGATION OF PARTICLES AND OF BACTERIA IN BODY FLUIDS.

In the course of experiments upon the reticuloendothelial system the preparation of Indian ink used was Watson's drawing ink which was found to be extremely stable in the presence of normal saline. But after intraperitoneal injections it was common to find heavy precipitates in the peritoneal cavity. Similarly after intravenous injections what appeared to be precipitates were found in the lung capillaries and the liver and spleen sinuses. It seemed that the blackness of the lung varied with the rate of injection and on one occasion a rabbit which died immediately after an injection was found to have a large precipitate of ink in the right ventricle. It was soon found that this preparation though remaining unaltered in sodium chloride was slowly precipitated by Ringer's solution, more readily by serum and defibrinated blood and almost instantaneously by whole blood and plasma. Gum Acacia was quite incapable of protecting the suspension against the action of the blood. With gelatin protection was afforded against the coarse precipitation produced by mixtures of equal parts of whole blood and protected ink. If however the ink was mixed in vitro with whole blood in the same concentration as it occurs in the animal after injection of about 5 c.c. and the mixture was incubated at 37°C. for 1 hour it was found that, though coarse precipitates were not formed,

yet the very fine state of division seen in the original ink had ceased to exist. The particles were aggregated together into microscopic masses. Now the same protected ink preparation when mixed with serum under the same conditions shows no alteration in dispersion of the particles. The stability of the suspension therefore is much less in the whole blood than it is in the serum. In the film from such preparations the small aggregates are partly independent and partly attached to clumps of platelets. Hardly any phagocytosis of the ink by polymorphs was observed though it undoubtedly occurred to a slight extent.

It is clear that the more nearly the fluid approximates to what is found in the blood stream the more marked is the instability of the suspension and the greater its tendency to precipitate. Yet the most actively precipitating of these fluids (plasma) is much less unstable than the blood probably is in the vessels with its capacity for clotting uninterfered with by chemical agents.

Similar observations to this have been made by Govaerts (1919, 1921) who also comments on the variation of effect of the blood of different animals, a matter which is also considered in some detail by Pfeiffer & Standenath (1923).

In considering the fate of such particles introduced into the blood stream the tendency has been to stress the phagocytic activity of the cells and neglect the phenomenon of precipitation. Now many writers have observed the occurrence of

massive deposits of ink, particularly in the lining capillaries following such injections (Lang (1926), Wislocki (1924), Nissen (1922), Boerner-Patzelt (1923), Kiyono (1914) and Westhues (1922)). Some authors have regarded these as simple precipitates while others regard them as having been brought to the particular situation by phagocytes which have subsequently disappeared (Westhues (1922)). My own preparations seem to suggest that the deposits are simple precipitates. The more unstable the suspension and the more rapid the rate of injection the greater is the tendency for this to occur and the more massive are the deposits. Further, according to Wislocki, the site in which they are found is determined to a certain extent by the route of injection.

I have injected into rabbits equal amounts of ink preparations, one of which was protected by 5% gelatin, the other being unprotected. The blood from the ear vein was collected by Bedson's (1922) technique for counting platelets. In the films ink could be detected for at least 2 hours in the case of the animal which received the protected preparation, whereas in the other no particles could be seen after 8 minutes. Further, the particles became much more rapidly aggregated into masses in the latter case and at a much earlier point of time. In both cases the aggregates adhered to the platelets which tended to mass together. Apparently the more unstable the suspension the more rapidly it is eliminated from the circulating blood.

Now a suspension of bacteria behaves in the main like

an emulsoid as regards precipitation. According to Bordet (1920 the effect of agglutinins is to convert the emulsoid suspension into a suspensoid condition which should then behave like the ink particles. Govaerts (1920) has pointed out that staphylococci when suspended in plasma or unheated serum from a normal rabbit in the presence of platelets flocculate readily, the bacteria aggregating on the platelets in virtue of the action of a "thigmophile" substance which le Fevre de Aric(1920) identifies with opsonins. Virulent pneumococci do not do this, but as le Fevre de Aric (1920) shows the phenomenon is observed if an immune animal's serum or plasma is employed. On repeating these experiments it was found that flocculation of suspensions occurred much more rapidly in the presence of platelets than in their absence and that the flocculation was earlier in the less stable fluids (plasma) than in the more stable (serum and heated serum). In my experiments the reaction ultimately occurred in all but whether this is observed or not depends on the activity of the fluid used and the time during which the observations last. The phenomenon seems to be very similar to the increased precipitation observed by Dean (1917) on the addition of globulin to a non precipitating mixture. These observations would suggest the possibility of greater activity of antibodies in vivo than in vitro, as suggested by Bull. It would also appear possible that the platelets, though not an essential part of the mechanism of removal of bacteria, may yet assist in it by helping in the formation of aggregates which must help to reduce the

numbers of organisms observed in the peripheral blood. It is one of a group of adhesive peculiarities conferred upon organisms by the blood of an immune animal, adhesion to one another (agglutinins) to leucocytes (opsonins), to endothelial cells (endothelial opsonins) and to platelets (thigmophile substance).

DISCUSSION OF RESULTS.

With few exceptions previous workers have found that bacteria introduced into the circulation have become considerably diminished in numbers in a short space of time. Govaerts (1 21, II) and Delrez & Govaerts (1918) remark that virulent pneumococci are not reduced in numbers when injected into rabbits. On the other hand Bull (1915, 1915, I., 1916 II.) and Singer & Adler (1924) show quite clearly that such organisms may be removed in large numbers for a certain time. Winternitz & Kline (1915) state that the organisms begin to grow immediately after their introduction but their observations as recorded do not permit of any conclusion as to what happens during the critical period. Reichstein (1914) makes a similar statement regarding haemolytic streptococci but Bass (1925), Hopkins & Parker (1918) and Bull (1917) report a marked preliminary elimination as in the case of pneumococci. As has been mentioned above considerable variations from animal to animal observed in the course of a large series of experiments, may be apparently due to individual peculiarities in the animal, as noted also by Singer & Adler. The effect of the virulence of the organism used has been found in these experiments to be quite definite, an avirulent strain being removed more quickly and completely than a virulent one. This agrees with observations made by Bull (1917). But with other organisms other results have been obtained (Teale & Bach, 1920).

The effect of immunization with either killed or living cultures is, according to my experiments, equally clear, the highly immune animal removing the organisms at a greater rate than the untreated one. Bull (1915) and Kitagawa (1914) agree that the immunized animal has the advantage in the case of the pneumococcus. Singer & Adler think tha power is conferred only to a slight degree by killed cultures as compared with living.

Hopkins & Parker (1918) state that this improvement does not occur in animals immunized against streptococci and Parker & Franke (1918) report that typhoid bacilli are not removed better by immunized animals than by normals. Bull disagrees with the latter finding but Bail (1905) has pointed out that while immunized animals do not dispose of typhoid bacilli more quickly than normals, the cholera vibrio is removed more rapidly if the animal has been immunized. Singer (1925) has also shown that anthrax bacilli disappear more quickly from the blood of the immunized animal.

Differences in this respect might very well be found according to the organism that is being used and in comparing the results obtained it is necessary to know the exact condition of the bacteria employed as regards its virulence and the exact stage of immunization of the animal. If the animal is highly immunized removal is rapid and complete but if the immunity has faded then the course of the septicaemia approximates

more and more to that found in the normal animal, differing from it only in degree. In the ordinary test for immunity, death or survival after inoculation of a dose of organisms, these fine shades are not seen. The result makes it appear that the difference is a sharp one but in these experiments it is seen at once that in many cases the difference is really very small and even two or three days after an injection it is quite impossible to tell what will happen. It takes very little apparently to turn the scale one way or the other.

The solidly immunized animal is distinguished from the normal by two main facts. The preliminary clearing is more rapid and more complete and the organisms once removed do not return to the circulation. In what does this preliminary improvement consist? Why is it that there is in the normal a rise in the number of organisms following the preliminary period of removal? In the answer to these two questions lies the solution of the problem of immunity against the pneumococcus and of its converse, the problem of the establishment of pneumococcal septicaemia. Previous work upon the problem of resistence against the pneumococcus has been almost entirely confined to that period when the animal is highly immunized. In this investigation I have endeavoured to see what is occurring at different stages, particularly when the resistence is fading. In the stage of high grade resistence the phenomena are exaggerated and therefore more easily observed; in the

stages of low grade immunity one may see if these phenomena are still to be observed, and if not which of them tend to survive. In other words in theearlier stages all the parts of the mechanism are displayed while later one may see whether all or any of these parts are essential.

To deal first with the mechanism; Bull in a long series of papers attempted to explain the whole of the removal of organisms from the circulation on the basis of agglutination or aggregation. If the animal were naturally resistent it agglutinated the organism (dog), if not it failed to do so (rabbit), unless the strain was an avirulent one. The improvement following immunization was due to the agglutinins produced. The benefit afforded by a protective serum was due to the agglutination which it effected. Hopkins & Parker could not confirm this in the case of streptococci. Other workers have failed to find evidence for the dominating significance of the agglutinins. Ten Broeck (1917) for example points out that Bull's conclusions are not applicable to the hog cholera bacillus. As shown above, I can find no close relationship between in vitro agglutination by serum and improved clearing capacity in the animal. That aggregation of this kind does occur in the highly immunized animal can not be denied but that it is the only or even the most important factor does not seem to be true. If it were true the diminution in numbers in whole blood in the test tube should bear some relation to what happens in the animal and I have shown this not to be the case.

Kitagawa (1915) experimented upon the reduction in numbers of organisms within the blood in a vein tied off from the general circulation and found that no reduction occurred there at all comparable to what happened in the circulating blood.

I have shown that improvement in capacity to remove organisms from the blood can be conferred passively upon a normal rabbit. The problem is therefore intimately connected with the problem of transference of antipneumococcal immunity. Now Eyre & Washbourn (1898) were unable to find any relationship between the agglutinating power of a serum and its protective action. Avery, Chickering, Cole & Dochez (1917) report similar results. Neufeld (1902) records that highly immunized animals may not have an agglutinating serum.

The only writer whom I have found to attach any importance to bactericidal action in the case of antipneumococcal sera is Romer (1902). On the other hand Eyre & Washbourn, Neufeld & Rimpau (1905), Wadsworth (1912, I.) and Armstrong (1925) agree in finding the bactericidal action of such antisera either absent or of very slight account.

Neufeld & Rimpau (1905) and Neufeld & Händel (1909) find in the opsonins or tropines the explanation of the protective action of the immune serum. They are supported in this view by Boehncke & Mouriz Riesgo (1915) and by Ungermann (1910). It is suggested however by Nunokawa (1909) that the assistance in phagocytosis is due to bacteriotropic influences combined with an anti-aggressive action. This view of an

indirect effect upon phagocytosis is supported by evidence brought forward by Tchistovitch & Yourevitch (1908) who describe the presence in virulent cultures of a soluble substance which hinders phagocytosis and which they term "antiphagine".

Wadsworth (1912, II.) states that in the normal dog there is practically no phagocytosis. If the animal is actively or passively immunized it can be observed; but in an animal examined long after immunization although still immune the phagocytosis may again be absent. These statements would appear to be based upon examination of the peripheral blood.

Nearly all this work has been done upon sera from a very highly immunized animal. Little work appears to have been done upon the animals possessed of low grade immunity against the pneumococcus. So far as the ordinarily recognized antibodies are concerned I have found their production irregular and their duration short. At the fourth day after immunization the agglutinins appear, and they are gone about the end of the third week. But some animals do not produce them at all after a single injection. In vitro phagocytosis is not seen in normal rabbit's blood (if a virulent organism is used); it is seen at the end of a week after immunization but is not usually observed after a month. Precipitins are irregular in their production, sometimes having disappeared by the end of a month, sometimes persisting as long as three months after a dose of living organisms. But the animal may still show a high degree of immunity long (months) after

these have ceased to be demonstrable. Wassermann (1899) reports the appearance of antibodies in the serum on the 3rd to 5th day and their disappearance on the 23rd day. The course of ordinary antibody production is then similar to what is found in the case of other organisms and is by no means coextensive with the duration of immunity. This condition of affairs, imm nity of the animal without the presence of demonstrable antibodies in the serum (agglutinins, precipitins) is frequently referred to as immunity in the absence of antibodies. Now the tests applied are very artificial and, from certain points of view, somewhat coarse. The animal is concerned with the elimination and localization of the invading organism and with the prevention or suppression of its power of multiplication. Armstrong (1925, I.) by a refinement of technique has been able to show that the duration of antibodies within the animal, as evidenced by protection for mice, is much longer than was usually supposed. It appears markedly increased three days after a single injection of killed culture and is still high at the end of a month. I have not repeated these observations but have found that there is still some evidence of protective power afforded to normal rabbits by 5 c.c. of serum from an animal immunized a month before even though precipitins and other antibodies are absent from that serum.

It has been shown that the presence of antibodies in very small amount, not demonstrable by the ordinary serological tests may be revealed if the organism be grown in

the presence of the serum or blood (the thread reaction of Pfaundler). This method has been utilized by Kinsella (1917) for demonstrating the presence of antibodies to the infecting streptotoccus in infective endocarditis. Kruse & Pansini (1892) observed that growth was irregular and often suppressed in the blood of an immunized rabbit, while it was unhampered in that of a normal rabbit. Virulent organisms grew better in the normal rabbit's blood and growth in the blood of the resistent dog and guinea pig was less marked than in that of the rabbit. Neufeld (1902) found that the serum of patients who had recovered from pneumonia frequently contained no agglutinins as ordinarily tested but that if pneumococci were grown in such serum the organisms were in masses. Sir Almroth Wright (1914) pointed out that the whole blood was often bactericidal when the serum or plasma showed no such effect. In 1916, Dochez & Avery studying the matter in more detail found that pneumococci grown in immune serum showed a long latent period and a diminished capacity for ~~xxxxxxxxx~~ certain metabolic activities usually associated with this organism. This they considered as specific inhibition of these activities and they termed it "Antiblastic Immunity". But Blake (1917) demonstrated that this phenomenon was simply due to the agglutinating growth which occurred in immune serum. Later Barber (1919, I. & II.) by means of the single cell method showed that there was no actual alteration in the rate of multiplication but the organisms tended to adhere to one another and remain

in chainsinstead of breaking up in the ordinary way. Consequently colony counts by plate methods did not really represent the growth activity of the organism and the delay in commencement of active growth was only apparent. Blake found that an amount of serum insufficient to produce agglutination could produce this phenomenon which was type specific.

Heist, Solis Cohen & Solis Cohen (1918) and Heist & Solis Cohen (1919) using capillary tubes coated with pneumococci into which whole blood was drawn without anticoagulant claimed to have demonstrated a bactericidal action on the part of the whole blood of an immunized animal. Further, avirulent organisms failed to grow in rabbit's blood and the blood of a naturally resistent animal (pigeon) was highly active. Bull & Bartual (1920) point out that the technique employed in this investigation did not permit of a distinction between destruction and inhibition of growth and further the variability in the type and consistency of the clot formed by the blood of the different animals was not considered. They themselves found that there was considerable variation in the growth inhibitory power of the blood of different animals as judged from the time which elapsed before the organisms became sufficiently numerous to be seen in films. They considered delay was to a certain extent due to phagocytosis and was roughly proportional to the amount of antibody present. Further the susceptibility of the various animals was indicated approximately by their capacity to delay growth.

I have employed blood treated with heparin as the preparation most closely resembling whole blood and yet not liable to coagulate. It appears from my experiments that an avirulent strain is readily destroyed by the blood of a normal rabbit or if it is not destroyed the growth curve shows a long latent period. On the otherhand a virulent strain of the same pneumococcus is capable after a latent period of about 2 hours of multiplying actively in such blood. If however the animal has been immunized the blood develops bactericidal properties which are of a low order but quite marked at the end of a week after immunization. The capacity to produce an apparent alteration of the latent period remains long after the ordinary antibodies have ceased to be demonstrable. I am not able to say if the length of the latent period is exactly proportional to the degree of immunity of the animal. But if there is any evidence of such an extension then the animal will be found capable of removing organisms from the blood at a greater rate than a normal rabbit and if it is marked the animal will usually be found to behave like a highly immunized one in this respect. In only one untreated rabbit have I so far found any such capacity for delaying growth and that animal when submitted to the test of an intravenous injection behaved like an immunized animal. This property of the blood appears earlier than the ordinary antibodies and has been demonstrated as long as 10 months after a dose of living organisms. It is clear

therefore that in the absence of the antibodies usually described evidence can be obtained of an altered condition of the blood resulting from the immunizing process. But it does not appear that this power is exactly parallel to the degree of immunity (capacity to dispose of bacteria injected into the blood stream). For in one case 11 months after immunization I have found a complete absence of this inhibitory power and yet the animal still showed evidence of enhanced clearing capacity as compared with a normal control. One experiment might be interpreted assuggesting that a certain amount of delay of growth due to immunization with a killed culture is associated with less capacity for eliminating organisms from the blood than a similar delay resulting from treatment with a living antigen. Are we then to assume that there is some other type of antibody or is this residual immunity which is found to exist to be attributed to an enhanced activity of the cells? Wadsworth thought that there was in all probability some form of antitoxic immunity but admitted that no toxin could be demonstrated. Of the existence of a toxin in the ordinary sense little evidence has been adduced. Cole (1912) claimed to have demonstrated in lysed cultures a toxic substance causing "anaphylactic symptoms" in guinea pigs. I know of no confirmation of this. Kruse & Pansini (1892) report the isolation of a similar substance from the blood of a septicaemic rabbit. Mennes (1897) described a fever producing toxin whose effect is neutralized by antiserum. Julianelle

& Reimann (1926) find that lysed cultures produce purpura in mice associated with diminution in the blood platelets.

Tchistovitch & Yourevitch (1908) as mentioned above considered "antiphagines", substances interfering with opsonic action, as one of the causes of virulence of the organisms. More recently Woo (1926) has shown that mixtures of serum and cells from a normal rabbit are inhibitory to the growth of avirulent pneumococci but not to that of virulent organisms. Sia (1926) has shown that extracts containing the specific soluble substance described by Dochez & Avery (1917) and Heidelberger & Avery (1923) when added to such mixtures confer on the avirulent organism the growth properties of a virulent organism. Certainly immunity can be conferred by an autolysate free from whole bacteria, as Wadsworth described and I have confirmed. From the histological observations made one cannot but conclude that phagocytosis is greatly increased in the immunized animal. Even at a time when it is not usually to be seen in test tube experiments the organs of the immunized animal show clear evidence of increased phagocytosis as compared with an untrested animal. It may be therefore that in these apparent cases of cellular immunity some such antibody is present.

In any case the destructive activity of the blood is by no means an accurate measure of the power of the animal to remove bacteria from the blood stream and it is necessary to take into account the part played by the tissues.

Bardach (1889) found that removal of the spleen diminished the natural resistence of the dog to anthrax and in 1891 he further discovered that after splenectomy rabbits became susceptible to the number one vaccine of Pasteur, which they normally withstand. This finding Melnikow Reswedenkow (1896) was unable to confirm. Wyssokowitsch (1886) considered that organisms injected intravenously were filtered out in the tissues. Werigo (1894) studying anthrax septicaemia found that phagocytosis was very active on the part of leucocytes in the capillaries of the lungs and the Kupffer cells of the liver. The spleen played a smaller part and he considered it to be the main focus for subsequent multiplication. Phagocytosis, he considered, occurred anywhere in the circulation where the bacteria were temporarily mechanically arrested. Final destruction was effected in the liver. Levaditi (1901) drew attention to the marked aggregation of polymorphs which occurred in the lungs after an intravenous injection of bacteria. Bartlett & Ozaki (1917, 1918), working with staphylococci in dogs observed the marked phagocytosis by blood cells in the capillaries of the lungs, liver and spleen. They observe that the mononuclears play a considerable part. Their view is that the bacteria are first arrested in the lungs and then passed on to the liver for final destruction. They express the view that in the fixed tissue cells (Kupffer cells, spleen cells) there is a mechanism which is complementary to the leucocytes and which can compensate for any deficiences in these cells. Nagao (1920) obtained

similar results with dead staphylococci. Ito (1917) observes the predominating activity of the lungs and liver and remarks that killed organisms (streptococci) appear to be dealt with by a normal animal in very much the same way as living organisms are dealt with by an immune animal.

Arima (1911) and Bordet (1895) observed that organisms were to be found by cultural methods in the organs in greater numbers than in the heart blood, the former considering the spleen and liver to play the main part in removal. Hopkins & Parker (1918) found that in the resistent cat the number of organisms (streptococci) isolated from the lung was large and also from the liver. In the normal susceptible rabbit the liver yielded many more colonies than the lungs but in the immunized rabbit the lungs were found to contain many more than in the normal. Parker & Franke (1918) in the case of the typhoid bacillus found 40 to 60 times as many organisms in the liver as in the other organs. The lung was more active in immune than in normal animals. My own observations have been confined to the first 10 minutes after inoculation. In that period the highly immune animal shows a most extraordinary degree of aggregation of organisms and phagocytosis by polymorphonuclear and mononuclear cells in the capillaries of the lung and in the liver. In the latter organ Kupffer's cells are actively phagocytic. The spleen shows little or no activity during this period. In the normal animal at this time these phenomena have not been observed but it is quite probable

that the mechanism is the same though slower in its action and less complete. I have found that the number of leucocytes in the circulating blood may be considerably reduced by benzole poisoning without any serious impairment of the eliminating power of an actively immunized animal, a fact which was also observed by Winternitz & Kline (1915). In the normal animal the effect is very similar though Winternitz & Kline find resistence to be diminished.

Similarly I have failed to make any significant alteration in the clearing power by means of injections of either Indian ink or lithium carmine, which were given with a view to blocking the reticulo-endothelial system. Singer (1925) on the other hand was able to produce interference with the later part of the elimination of anthrax bacilli by such means and Singer & Adler (1924) found the resistence to Type III. pneumococci greatly diminished by such injections.

I have already referred to the experiments of Bull & McKee (1922) and Govaerts (1921) showing that removal of the platelets from the blood does not diminish the rate of removal of bacteria subsequently injected.

It would appear that the mechanism of removal is a complex one and not confined as some authors maintain to any one phenomenon. It is suggested that the humoral part of the process consists in the production of an increased adhesiveness of the bacteria as a result of which they tend to stick to one another, to leucocytes, to endothelial cells and

to platelets. In addition active phagocytosis by the polymorphonuclear and mononuclear cells of the blood and the cells lining the sinuses of the liver and possibly also of the spleen plays its part. And it would seem that either of these groups of cells may make good very considerable deficiencies in the other. In addition to which it seems highly probable that the number of cells at the disposal of the animal is much in excess of requirements. It has a great surplus on which to call. So far as the pneumococcus is concerned the factor which influences the rate of removal most markedly is the process of specific immunization.

The question of the occurrence of a purely cellular immunity after the obvious humoral antibodies have disappeared has been discussed above. Singer & Adler (1924) maintain that the whole of the immunity to Type III. pneumococcus is of this nature and can never be transferred by means of serum, a statement which does not agree with the findings of other workers. But they were unable to demonstrate any increased capacity on the part of the perfused liver of an immunized animal to remove pneumococci from the perfusate as compared with livers from normal animals. Hahn & von Skramlik (1919) and von Skramlik & Hunermann (1920) found that perfusion of the liver with B. coli antiserum conferred on that organ an increased capacity to remove the homologous organisms even after the organ had been well washed out with perfusate. But Manwaring & Coe (1916) attributed such results to traces of serum still

remaining in the organ. They found that serum in a concentration 100 times less than that which produced agglutination was able to increase very markedly the capacity of the liver cells to remove pneumococci from the blood (endothelial opsonins). In discussing the existence of any such form of immunity it must be borne in mind that the amount of antibody required to alter the state of resistence is in all probability extraordinarily small and might well escape detection by our present methods. I am not satisfied that any clear evidence for its existence has been supplied in the case of the pneumococcus. Even in the case of a normal rabbit Armstrong has been able to demonstrate a small amount of protective antibodies. In one relatively resistent animal 11 months after immunization I have failed to demonstrate antibodies in any greater amount than in a normal rabbit.

Some authors have sought the explanation of slightly enhanced resistence in a specific hyperleucocytosis. I have already shown that very marked alterations in the leucocyte count in the peripheral blood may be produced without any obvious effect on the capacity to remove organisms from the blood. Further, my experiments revealed no evidence of the occurrence of such a leucocytosis. The only difference observed was a rather more rapid onset of leucopenia in immunized animals as was reported by Andrewes (1910). This presumably suggests rather more rapid aggregation of the leucocytes in the capillaries of the internal organs, especially the lungs - a sort of visceral leucocytosis.

Nor have I observed any marked alteration in the temperature reaction of the immunized as compared with the normal rabbit. Both tend to show a rise of temperature after about 2 hours. The experiments already recorded seem to show that the pneumococcus is not markedly interfered with in growth by the temperatures ordinarily found in the rabbit but may be slightly impeded by a marked hyperpyrexia.

But the capacity to get rid of pneumococci more quickly is not the only nor even the most important point of difference between the normal and the immunized rabbit. In the immunized animal the removal is definitive while in the normal the reduction is only temporary and tends to be succeeded by an increase. Bull (1916) has suggested that the pneumococci present during the period of increase are less agglutinable and less susceptible to opsonization than those isolated during the preliminary drop or the second fall. So far as can be gathered these observations were made upon cultures of such organisms and not upon the actual organisms present in the blood. Hopkins & Parker have questioned this explanation and found that if septicaemic blood is drawn off and injected into a fresh animal the organisms are rapidly removed from the circulation which they think could not happen if the organisms were significantly modified. I have repeated this experiment in the case of pneumococci with similar results but agree with Hopkins & Parker that the experiment is not conclusive as the dose administered is of necessity very much smaller than what is usually used in inoculations of

That the clearing capacity of the animal is by no means exhausted at the time when the septicaemia is present has been shown for streptococci by Hopkins & Parker who injected a dose of culture into an animal in this condition and found it quickly removed, the grade of septicaemia being frequently less, though not constantly so, a few hours after than it was before the injection. Bull (1916) also noted this and I have confirmed the result. The existing septicaemia goes on as though no injection had been made. Bull suggests that this means that the new organisms are readily removed but the organisms constituting the septicaemia are resistent to such removal. Hopkins & Parker think that organisms are removed and destroyed chiefly in the viscera - especially the liver - but in the normal animal many organisms are to be found in cultures from other organs such as muscles in which they multiply. The septicaemia is kept up by multiplication in these foci. Organisms are constantly being removed but they are also constantly being replaced by others. The balance constitutes the septicaemia. Bartlett & Ozaki (1918) observed that in an animal almost dead of staphylococcal septicaemia marked phagocytosis of a fresh injection still occurred though the leucocytes seemed to be less active than the fixed tissue cells. They also note (1917) collections of unphagocyted bacteria in certain organs, especially the kidney, which they look on as foci of multiplication of organisms that have developed a special resistence; the period of delay before increase occurs is a period of

adaptation. Werigo had this same idea of foci of multiplication in regard to anthrax septicaemia. He considered them to be located in the spleen. Meyer, Neilsen & Feusier (1921) and Parker & Franke (1918) also mention such foci in the bone marrow in the case of experiments with B. typhosus and allied organisms. Kruse & Pansini suggested the secondary rise in the number of organisms was due to growth of organisms through the capillaries into the veins. Singer (1925) and Singer & Adler (1924) suggest that the immune animal's cells take up the organisms and destroy them whereas the cells of the normal animal may take up the organisms but fail to destroy them and are instead damaged themselves and become foci in which multiplication may occur. Bull (1915) suggests that masses may be produced so large as to be unphagocytable and thus be sources of renewal of the septicaemia. In addition there are certain statements in the literature (Muir & Ritchie) to the effect that in a septicaemia the organisms are actually multiplying in the peripheral circulation. There is, so far as I am aware, only one reference to the state of growth in which an organism is found in pneumococcal septicaemia - Barber remarks of one strain with which he worked that it was apparently not actively multiplying in the blood of the patient from whom it was isolated.

The experiments recorded above upon the subject seem to throw some considerable light on this point and to explain certain features of the experimental septicaemia. The organisms in a twenty-four hour culture of virulent pneumococci as

employed in these experiments are in the phase of latency. In rabbit's blood or serum broth this latency lasts for a period of two hours at 37°C. and about the same time at the temperature of the rabbit's body. Now the samples of blood drawn one hour after inoculation of a rabbit with such a culture show that the organisms within it are still in the period of latency and remain so for one hour. After that period the organisms in vivo are in the stage of active multiplication for at any rate 2 - 3 days. During the period of active reduction then the animal is working upon a non-multiplying or at any rate non-increasing organism. After two hours the conditions are altered. The rise in numbers in my series of experiments with such cultures has never occurred before the end of the two hour period. At the same time it is frequently later, especially in the cases where the animals ultimately survive. This seems to me to be due to the fact that the measurement of the septicaemia is a measurement, not of the actual amount of growth which occurs but of the balance between growth and elimination. The growth in the animal even during the period of increase is practically never at the same rate as in the blood in the test tube. There is a definite brake on it. Further, much of the multiplication may be occurring in foci in capillaries or cells in distant parts of the body and therefore possibly secluded from the general circulation producing its effects on the numbers of organisms only after some time.

If this be true it would be expected that the use of an actively growing culture would result in a marked alteration of the course of the experimental septicaemia. If such a culture is injected intravenously the preliminary period of decrease in numbers may be entirely suppressed the organisms showing a fairly steady increase straight on from the time of inoculation. It would be expected that in certain cases such a steady increase from the beginning might not be found. Presumably even in these cases some organisms are being removed but multiplication more than makes good the loss. In others the amount of removal might be greater and the curve correspondingly altered.

It is interesting to observe that there appears to be a fairly close resemblance between the course of growth as seen in the rabbit's blood in the test tube and that which is found to occur in the animal so far as these two periods - latency and active multiplication are concerned.

There would seem now to be a clear basis for the explanation of the higher virulence for mice of pneumococci in the period of active growth than in that of latency (Felton & Dougherty, 1924). The effective dose in the former case is the number of organisms inoculated, in the latter it is the number surviving the latent period, whatever that may be in mice.

The culture in the latent phase of growth when inoculated is to all intents and purposes an inoculum of foreign

bodies and so far as possible dealt with as such, in a normal animal rather like an extremely well protected suspension somewhat slowly removed, in an immune animal like an ill protected one susceptible like it to humoral influences and subsequently phagocyted. After the latent period has passed the resemblance ceases to exist. The organisms, if not destroyed, are now multiplying and the septicaemia develops according as there is a balance between rate of multiplication and rate of destruction. Anything therefore which prolongs the latent period increases the time during which unhampered elimination may occur. A rise in temperature does this in the test tube. It is possible it may do so in the animal though the evidence I have obtained is not conclusive. But once multiplication has begun a rise of temperature appears to be of little assistance.

Now it has been demonstrated above that blood from an immunized animal also prolongs the latent period as judged by colony counts. For reasons already discussed it is not permissible to assume that this necessarily implies an actual suppression in increase in organisms. It may be simply a prevention of dispersion. The cocci may be increasing in numbers but not becoming detached from one another. The part played by immunization is probably much more related to its capacity to enhance phagocytosis and favour localization. Tsuda (1923) points out that histologically the characteristic feature in the immune animal is that the organisms are localized.

Such localized growth would provide the leucocytes with a fixed point of attack instead of an ever increasing, ever moving one. Further increase in size is up to a point favourable to the chance of phagocytosis occurring.

As regards intracellular destruction of organisms, as opposed to ingestion or phagocytosis, practically nothing certain is known. The latter does not necessarily involve the former. It is possible that this might be enhanced in the immune animal and account for cellular immunity but I have no information to justify any such assumption.

I have pointed out (1925, 1926) that in the septicaemia occurring in subacute bacterial endocarditis in man and in experimental endocarditis in rabbits the organisms are incapable of multiplying in the blood of the patient and are certainly in the phase of latency. Clearly then there are two distinct types of septicaemia, and it would be interesting to know which of these types is found in the various forms that occur in man. Such information might conceivably be of value from the point of view of prognosis and treatment.

SUMMARY.

1. Virulent pneumococci inoculated into a normal rabbit undergo rapid removal for a short period but subsequently increase in numbers.

2. Avirulent pneumococci are removed more rapidly and do not reappear in the blood.

3. Immunization enhances the capacity of the animal to remove virulent organisms and prevents their reappearance.

4. This improvement can be detected to a slight extent five hours after injection of killed culture, but becomes marked three days thereafter. It has been observed to last as long as ten months after immunization.

5. The outstanding effect of immunization with Type I. pneumococci is the enhancement of activity of the body fluids favouring phagocytosis but the existence of a slight residual purely cellular immunity cannot be altogether excluded.

6. Agglutinins, opsonins, precipitins, complement fixing antibodies do not appear to be essential as such to this improvement.

7. The blood of a normal rabbit is bactericidal to avirulent pneumococci or inhibits their growth. It has no such action upon virulent pneumococci.

8. Blood of an immunized rabbit is destructive to virulent pneumococci or delays their growth. The property of delaying the commencement of growth appears earlier and lasts far longer than any other antibody activity tested.

9. The destructive action of the blood is of a much lower order than that of the animal as a whole.

10. The leucocytes may be considerably reduced without interfering with the capacity of the animal to dispose of organisms introduced into the blood.

11. "Blocking" of the reticulo-endothelial system has proved similarly ineffective.

12. This is thought to be due to the complexity of the clearing mechanism involving as it does a variety of phenomena of adhesion and phagocytosis by mutually complementary systems of cells.

13. Virulent pneumococci injected in the phase of latency behave in vivo as in vitro. The increase occurring some time after injection is due to their passing into the phase of active growth.

14. Virulent pneumococci introduced in the active phase of growth do not undergo the preliminary rapid removal.

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