AN EXPERIMENTAL STUDY OF THE NEISSER-WECHSBERG PHENOMENON.

BEING PART OF A THESIS ACCEPTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN THE UNIVERSITY OF LONDON.

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HISTORICAL.

During their study of quantitative estimation of immune bodies in bactericidal immune sera, Neisser and Wechsberg (1901) made a curious observation which since then has been generally known as "Complement blocking (Komplement Ablenkung)" or as the "Phenomenon of Neisser and Wechsberg." Working with an immune serum against *Vibrio metchnikovi* obtained from a rabbit by repeated inoculations of the organisms, they observed that while medium doses of the inactivated serum showed a complete bactericidal action against a certain dose of homologous bacteria, in presence of a definite dose of complement, larger and smaller doses had either a considerably weaker action, if the bactericidal function was completely in abeyance, though the doses or complement and bacteria employed were still the same. In other words, paradoxical as it may seem, larger doses of immune serum were found to be positively harmful.

The authors attempted to explain this on the basis of their surplus amboceptor theory; since in tubes clearly showing this effect a large amount of amboceptors were apparently present, in comparison with the amounts of bacteria and complement added. Supposing that bacteria absorbed considerably more of this antibody than was actually necessary to bring about their lysis, a still larger amount was left over, free in the fluid. On subsequent addition of complement, these amboceptors united readily with some portion of it, seeing that they contain a complementophile group. Some of the complement, which ought to have united only with the antigen-amboceptor complex is thus deviated. The amount of complement left available to unite with such complexes, was insufficient to bring about a complete bactericidal effect, hence the occurrence of inhibition. With increasing dilutions, no such deviation can occur since amboceptors are not present in excess, and a complete bactericidal effect thus becomes manifest.

It was suggested that the chief factor in the production of the phenomenon was agglutination and the mechanical effect of the clumping prevented the bactericidal effect.

Lipstein (1902) produced two sera with strong agglutinating power against *Vibrio metchnikovi*—one from a goose, the other from a goat. Both sera had the same agglutinating titre, viz. 1–1000. Using rabbit's serum as complement, he showed that whilst inactivated immune serum from the goat showed distinct Neisser-Wechsberg phenomenon, it was entirely absent in the case of the corresponding goose serum. Agglutinins therefore were not concerned in the action; and as it was possible to remove completely the inhibiting action by absorption with the homologous bacteria, the amboceptor nature of the inhibiting bodies he holds as proved and generally supports the conclusions of Neisser and Wechsberg.

Brekke (1916) studied the occurrence of the phenomenon in sera obtained from patients suffering from enteric fever. He did not find "inhibition" a constant attribute of all the immune sera, even when employed in large doses, and possessing a high bactericidal titre. Inhibition was observed in only 22·5 per cent. of cases. In view of his observations, therefore, Brekke believes the phenomenon is not a specific one, but is produced after inactivation of the serum, *i.e.* by the action of complementoids.

Thjøtta (1920), however, has made an interesting contribution to the subject. After examining various factors influencing inhibition he comes to the conclusion "that inhibition is due to antibodies that arise during immunisation, or during natural disease. These antibodies are specific inhibiting antibodies, which combine with dissolved antigen to form molecular complexes, which have a marked tendency to absorb complement, and thus to withdraw it from bactericidal antibodies." In other words, his specific inhibiting antibodies act like "anti-complements." He also showed that if *active sera* (*i.e.* with their complement intact) of immunised animals are examined (without the addition of foreign complement), complete abolition of normal bactericidal action takes place. He also believes this "inhibiting faculty" to be beneficial to the animals, as it enables them to fight the invading organism.

INTRODUCTION.

As the conclusions arrived at by Thjøtta were considered to be very important (if they be true) and not at all in accordance with our present conception of what happens in immunised animals, the present investigation was undertaken to examine in detail some of the points brought forward by him and also, if possible, to elucidate further the mechanism underlying this phenomenon.

The subject has been approached from three different standpoints. Firstly, it was thought desirable to take up the phenomenon as met with in Bacteriolysis. While it is true that it is very difficult to study adequately all the underlying principles, and especially their quantitative relations, while working with such

living cells as bacteria, so far as this phenomenon is concerned it in fact furnishes many advantages. It is comparatively easy to produce bacteriolytic immune sera, and some of the features as to origin and progress of the phenomenon can be well studied and followed, and questions such as specificity can also be considered.

Secondly, the conditions governing the phenomenon in Haemolysis are studied. A discussion concerning the nature and causation of the anomalous effect seen in the Neisser-Wechsberg phenomenon is taken up later, and an attempt is made to examine critically the various explanations which have been offered from time to time. Lastly, the question is raised whether it is possible to encounter inhibition *in vivo*—*i.e.* in actual treatment of disease with immune serum.

The results which have been obtained are therefore recorded separately under these headings, notwithstanding the incidental risks of repetition, which such an arrangement involves.

TECHNIQUE.

Rabbits were immunised by inoculating them intravenously with increasing doses of cholera vibrios, at stated intervals. These organisms were selected, because it was known that cholera vibrios undergo lysis easily, and are therefore particularly suited for the study of the Neisser-Wechsberg phenomenon. Three strains were used:

I. Italian strain (used by the Italian Government for making vaccines during the war).
II. R.A.M.C. No. 4, and
III. R.A.M.C. No. 6.

For all these we are indebted to Dr Douglas.

Throughout all our experiments with the immune sera so obtained, we have followed for the investigation of bacteriolytic action the method originally described by Neisser and Wechsberg. Preliminary titrations of the normal sera were always made prior to immunisation. The following will serve as an example of such a titration:

Exp. To different quantities of normal active serum 1 mm. loopful of 1–10 dilution of 48 hrs old cholera culture was added; two drops of broth were then added to make the whole into a suitable medium for growth. Total volume was made up to 0·5 c.c. with 0·85 per cent. saline solution. Tubes were incubated from an hour to an hour and a half, after which time 1 mm. loopful was inoculated on agar, and the number of colonies counted on the next day. Adequate controls were also put up.

The results of such an experiment are presented in Table I.

For the development of bactericidal action, during immunisation, the same experiment was repeated in all its details except for the substitution of an immune serum instead of the normal one. The results are shown in Table II.

It will be seen, that in both experiments, the serum was not inactivated, thus using its own native complement for the bactericidal action. Reference will be made to this fact later.

Table I.

Showing bactericidal action—normal serum.

Tube	Normal serum dilution in saline	Cholera vibrios	Broth drops	Results. Remarks
1	0·3	1 loop (1 mm.) of 1–10 dilution of 48 hrs old broth culture	3	No colonies
2	0·2		3	,, ,,
3	0·1		3	,, ,,
4	0·05		3	Few ,,
5	0·025		3	Many ,,
6	0·0125		3	Hundred colonies
7	0·0062		3	Several hundred colonies
8	0·0031		3	,, ,, ,,
9	0·2	—	3	None (blood control)
10	none	—	3	None (saline control)
11	,,	1 loop	3	Abundant growth (control)

0·1 c.c. shows complete bactericidal activity, while no inhibition is seen with higher doses.

Table II.

Showing bactericidal action—immune serum active.

(Tested fifth day after second injection.)

Tube	Immune serum dilution in c.c.	Antigen	Broth drops	Results in colonies	Remarks
1	0·1	1 loop as in previous experiment	3	None	Serum not inactivated
2	0·05		3	,,	
3	0·025		3	,,	
4	0·0125		3	,,	
5	0·0062		3	,,	
6	0·0032		3	,,	Controls as before
7	0·0016		3	,,	
8	0·0008		3	Few	
9	0·0004		3	Many	
10	0·0002		3	Several	

Though bactericidal titre has increased, inhibition does not occur with fresh (active) serum.

For the investigation of the Neisser-Wechsberg phenomenon, the method adopted was almost the same, but the immune serum used was previously inactivated by heating to 55° C. for $\frac{1}{2}$ hour. Foreign complement (that of a normal rabbit in almost all cases) was added in a suitable dose. The activating power of such a normal serum was previously determined by adding different quantities to a constant dose of amboceptor and antigen.

The following may be taken as an illustration of a typical Neisser-Wechsberg phenomenon.

When reading the results of such an experiment, I shall have occasion to allude to what is known as the "Inhibiting Titre." Thjøtta employed the term for that dilution of inactive immune serum which shows some amount of inhibition, though accompanied by some bactericidal action; beyond this

dilution, there is a total bactericidal effect. I believe it would perhaps be advisable to take that dose of the immune serum, as an "inhibiting dose," in which (if we may say so) bactericidal action is in complete abeyance. Thus in Table III the inhibiting titre is 0·2 c.c., while its bactericidal titre may be taken to be somewhere between 0·0016 and 0·0008.

Table III.

Showing the Neisser-Wechsberg phenomenon.

Tube	Inactive immune serum in c.c.	Complement in 0·1 c.c.	Antigen	Results in colonies	Remarks
1	0·3	0·005	1 loop as in previous experiments	∞	Controls were put as usual
2	0·2	„		∞	
3	0·1	„		Many thousand	Total volume in all 0·5 c.c.
4	0·05	„		25 (?)	
5	0·025	„		Several hundred	
6	0·0125	„		„ „	
7	0·0063	„		Few hundred	
8	0·0032	„		50	
9	0·0016	„		50	
10	0·0008	„		10	
11	0·0004	„		8	
12	0·0002	„		100	

Attention may here be called to the close approximativeness of the bactericidal titres as investigated by these two methods (Tables II and III). What experimental error may be introduced by using the "loop" method for introduction and plating of bacterial culture may be considered negligible.

Appearance and progress of inhibition during immunisation.

It will be seen from Table I, that the inhibitory function is entirely absent in normal sera. A reference will be made to this aspect of the question later. During immunisation, however, this faculty is soon attained. In one of my experiments, inhibition was distinctly shown by serum taken four days after the first injection; in other words it may be stated that as soon as a serum begins to develop increased bactericidal activity, the phenomenon of inhibition also manifests itself. It was in fact an observation of this nature which led Thjøtta to believe in the existence of a special "inhibiting antibody" which is produced in the body simultaneously with the development of bactericidal amboceptors. That this assumption is not necessarily true will be shown subsequently.

The progress of this inhibiting faculty however does not always run "*pari passu* with the bactericidal one." In some cases an immune serum never attains the inhibiting action at all, as shown by Thjøtta with artificially prepared immune sera against *B. typhosus*, while it is comparatively easy to obtain that result with either the dysentery or cholera bacilli. During the progress of immunisation, no definite relation between the two functions can be found. Sera with high bactericidal action show weak inhibiting function

and *vice versa*. During immunisation of one of the rabbits, I tried to follow the progress of this inhibiting property for a considerable period. The results are recorded below in Table IV.

It will be seen that after the second injection, inhibition was so marked that nowhere in the series of tubes put up was complete bactericidal action found. After the subsequent injections, however, complete bactericidal effect was quite evident at first in two or three tubes and then in many, as the bactericidal titre increased. The inhibition titre however falls, as is shown by the fact that after the fifth injection even with 0·4 c.c. of immune serum complete suppression of bactericidal activity was not met with. In short, the Neisser-Wechsberg phenomenon tends to disappear.

Table IV.

Showing the progress of "inhibition" during immunisation.

Quantity of amboceptors in c.c.	Before immunisation		After 2nd injection		After 3rd injection		After 5th injection		Remarks
	B	I	B	I	B	I	B	I	
0·4	0	—	0	...	0	...	Complete bacteriolysis with all	...	Rabbit immunised as under:
0·3	0	—	0	...	0	...		Many	
0·2	0	—	0	...	0	Many		125	1st inj. 0·05 agar slant (dead)
0·1	0	—	0	Many thousand	0	Few hundred		75	2nd inj. 0·03 living
0·05	25	—	0	25(?)	0	50		15	3rd ,, 0·07 ,,
0·025	Many	—	0	Several hundred	0	10		0	4th ,, 0·1 ,,
0·0125	Hundred	—	0	100	0	0		0	5th ,, 0·1 ,,
0·0064	Several hundred	—	0	25	0	0		0	Antigen, 1 loop *V cholerae*.
0·0032	...	—	0	25	0	10		0	Complement, 0·005 c.c all through.
0·0016	...	—	0	10	0	20		0	No "inhibition" with fresh active sera.
0·0008	...	—	25	5	0	Many		0	*Note.* B = Bactericidal.
0·0004	...	—	Many	Many	Many	,,		0	I = Inhibiting.
0·0002	...	—	Several hundred	,,	,,	,,		0	Results in colonies.

The same effect was strikingly seen during the immunisation of another rabbit, as the following table will show.

Table V.

Progress of inhibition during immunisation.

Amount of amboceptor	After 2nd injection	After 4th injection	After 5th injection	After 6th injection
0·4	...	...	Many	10
0·3	Many thousand	Many	4 (?)*	3
0·2	Several hundred	90	Many	0
0·1	22	10	1	0
0·05	15	0	0	0
0·025	4	0	0	0
0·0125	—	0	0	0
0·006	—	0	0	0

* Zone phenomenon (see p. 420).

Conditions met with after the second injection are markedly different from those after the sixth, when inhibition is so exceedingly weak.

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Is the phenomenon observed with active serum?

Thjøtta answers this question in the affirmative. Yet, as will be seen from Table IV in my series of experiments, not once was it possible to observe any inhibiting effect, making use of the native complement from the immune serum itself. This of course means that the serum was not inactivated as heretofore. In view of these contradictory results it is necessary to examine in detail some of the theoretical considerations advanced by Thjøtta. According to him an immune serum which is able to inhibit the function of a foreign complement, is able also to bring about the same action against the complement of the immune serum itself. Such a serum can never show any bactericidal action, whatever the amount of serum employed, without the addition of foreign complement.

The relation of complement to "inhibition" I will discuss in detail in the following section, when I have tested, as far as this phenomenon is concerned, the efficiency of complements from different sources. There I had incidentally used the complement from the immune rabbit itself, *i.e.* a complementing dose of the same fresh immune serum was added to usual quantities of inactivated serum. The result will be clear from a reference to Table VI. Inhibition was distinctly less marked with this, than with any other complement, *i.e.* quite in contradiction to what Thjøtta believes. This result, also, is quite in harmony with some other facts of complement action observed by other workers. Thus, according to Muir and Browning (1905), "highest dosage of the complement of an animal is required, when used against its own corpuscles." This means that a complement exerts a sort of protective action for the benefit of the host from which it is derived. It is therefore natural that inhibition should be found weak with the homologous complement, detrimental as the phenomenon is to the animal.

These findings with regard to the appearance and progress of inhibition (as discussed previously) differ entirely from the conclusions arrived at by Thjøtta. Inhibition according to him increases and not decreases as immunisation proceeds, with the result that during this process the serum of the animal loses what little bactericidal power it possessed previously. He therefore believes that the bactericidal property plays no part "in the stable immunity after disease." Now as the organism is decidedly immune to the infection for some time at least after it has suffered from it (in the majority of cases, at any rate), are we to attribute this state to the inhibiting function developed in the serum? Thjøtta suggests that in an immune animal, with a well developed inhibiting function, endo-toxins are only slowly liberated, thus allowing other immune forces to come into play before real intoxication results.

The following factors make this explanation unacceptable:

(1) Inhibition does not kill bacilli. They may thus continue to grow in body fluids and ultimately prove harmful to the host.

(2) Inhibiting faculty ought to last longer than the bactericidal one.

Thjøtta's own experimental results disprove this. An immune serum, examined one year after it was removed, had lost almost entirely its inhibiting action, while total bactericidal effect was not much diminished. I have observed this *in vivo*. In one of my rabbits examined 4 months after the last injection the phenomenon of inhibition, once very marked, had entirely disappeared.

(3) During immunisation, animals showing strong inhibition must always tolerate inoculations with higher doses of bacteria better. However it is known that inhibition may be the cause of sudden death. Neisser and Wechsberg quote a statement of Pfeiffer: "It has frequently happened to me that highly immunised guinea-pigs died after an injection of moderate amount of virus. On section there were found in the peritoneal cavity living vibrios, sometimes in considerable numbers."

It may therefore be concluded that inhibition cannot afford any sort of protection to the animal, during or after infection.

Complement and inhibition.

The experiments of Lipstein have already been referred to. He was unable to produce the Neisser-Wechsberg phenomenon with an immune serum against *Vibrio metchnikovi* obtained from a goose using rabbit's serum as complement. He could, however, obtain inhibition distinctly when he used goat's complement instead. Sera from different sources have been known to vary greatly in their complementing power. It was thought desirable to study the effect of complement from different sources as regards the present phenomenon. The following table is a record of some of the experiments which have been done with this object in view:

Table VI.

Showing the effect on inhibition of complement from different sources.

Tube	Quantity of immune serum (rabbit A) c.c.	Immune serum of rabbit A as complement	Complement of another rabbit immunised with the same strain	Normal rabbit complement	Human	G.P.
1	0·4	Many	95	Many	350	Many
2	0·3	13	68	84	180	85
3	0·2	9	25	10	140	11
4	0·1	—	—	—	60	8
5	0·05	—	—	—	—	—
6	0·025	—	—	—	—	—
7	0·0125	—	—	—	—	—

The doses of antigen and complement were the same in all cases. Effect of using the complement from the same serum is shown; this action having already been explained in the last section.

The degree of inhibition entirely depends on the dose of complement employed; utilising 0·005 c.c. as the dose of complement, inhibition was far more marked than with 0·05 c.c. as the dose. It may therefore be possible to suppress the inhibiting action by employing larger doses of complement. The neutralisation (if we can so speak of it) of the inhibiting effect does

not proceed in definite proportions. Experiments were done to study this point. Complement is added in different doses, and the results are compared to those obtained when the whole amount was added at once. The details will be clear from the following tables, VII and VIII.

Table VII.

Showing the effect after addition of complement in different doses.

Tube	I	II	III	IV	
Amount of amboceptor	0·4	0·3	0·2	0·1	Remarks
1st series	Colonies	Many thousand	23	13	Complement—0·025 in 0·1 c.c. Antigen—2 mm. loop of 1–10 dilution of broth culture
After plating some amount from above, further amount of complement, 0·025 in 0·1 c.c., was added. Tubes incubated again and plated.					
2nd series	...	9	0	0	Result when total complement (0·05) was thus added in two lots

Table VIII.

Result after addition of complement in still smaller fractions.

Tube	I	II	III	IV	
Amount of amboceptor	0·4	0·3	0·2	0·1	Remarks
1st series	...	...	...	...	Complement added—0·02 Antigen—same as last, incubation 1 hour
Add further amount of 0·02, and then again after 30′ 0·01. Incubate and plate again.					
2nd series	...	...	120	13	Total amount of complement added—0·05, but in three lots
	...	...	40	1	0·05 complement. Added all at once

It is clear therefore that the results are analogous to the adsorption phenomena met with during neutralisation of toxin and antitoxin. It is likely that the phenomenon of inhibition depends on some physical factors. Perhaps the molecular constitution of the inactive serum is changed by the addition of fresh serum as complement, and the result is different when it is added in different quantities. It must be admitted however that such explanations are mere conjectures.

Study of inhibition in haemolytic experiments.

Neisser and Wechsberg failed to observe the "blocking of complement" during their experiments with haemolytic sera. This negative result they attributed to the difference in the affinities of the reacting bodies. Ehrlich (1902) also adopts the same standpoint, pointing out the impossibility of correctly understanding the phenomena of immunity without the assumption that "certain haptophore groups become increased or decreased in their chemical energy, owing to changes in the total molecules." Thus the affinity of haemolytic antibody for the receptors of the red blood corpuscles is directly

increased; when the antibody molecules are acting in excess, complement cannot unite with them alone, and complete haemolysis is brought about.

Questions of affinity are indeed difficult of experimental investigation. It may however be pointed out, if the above explanation is true, what happens here is in direct opposition to what takes place in a bacteriolytic system. It is difficult to understand, that while there is such a complete parallelism between the action of bacteriolytic and haemolytic amboceptors in other respects, their action should be so diametrically opposite in this respect only, in spite of similar concentration of the reacting molecules.

The phenomenon in haemolysis has been chiefly observed by Gay (1905, II) and Sormani (1916). The former had observed (1905, I), that if a haemolytic immune serum, inactivated by heating to 55° C., was brought in contact with the homologous corpuscles, and the latter removed later by centrifugalising, the supernatant fluid (*i.e.* the "treated" serum) had acquired the property of fixing alexine. This he found out was the result of a specific precipitate, formed by contact of the immune serum (precipitin), and a remnant of the native serum carried by the red blood cells along with them (precipitonogen). In haemolytic mixtures precipitin and precipitonogen being present in sufficient relative proportions, the resultant serum precipitates "deviated" some of the complement, thus giving rise to the phenomenon of inhibition.

These views of Gay have been contradicted by Sormani (1916). He produced such specific precipitates in quantity. After addition to a mixture of sensitised red blood cells and complement, complete haemolysis occurred, and no diminution in the activity of the complement was met with. He also observed that sera with a high precipitating titre and a weaker haemolytic one showed the inhibitory effect better than sera with a weak precipitating titre but a high haemolytic one. Microscopic examination of the centrifugalised corpuscles from inhibiting mixtures revealed them as shrunken and jagged. He therefore suggests that the precipitating action of the serum has a direct bearing on the Neisser-Wechsberg phenomenon. It does not act, however, by forming a specific precipitate which absorbs complement as suggested by Gay but rather it has an action on the surface of the antigen, which lessens its solubility. He also showed that if corpuscles were suspended in a very strong concentration of immune serum, their surface was so much altered that gentle mechanical shaking alone brought about their haemolysis without even the subsequent intervention of complement. (Phenomenon of specific fragility—"specifische Sprödigkeit.")

During the following haemolytic experiments I used an immune serum against sheep corpuscles obtained from Messrs Burroughs Wellcome & Co. Such an immune serum will be designated hereafter as "amboceptor" for convenience. It need scarcely be added that before each experiment the amount of complement to be employed in the test was always determined previously, by the usual way of complement standardisation. The usual series of tubes was then put up, with a fixed dose of complement, with a fixed quantity of homologous

corpuscles but with a varying amount of amboceptors; and after suitable incubation, results as to occurrence or absence of haemolysis were recorded.

It was found that inhibition could be observed with haemolytic sera, but not so readily as in bacteriolytic experiments, and under certain conditions only as regards the doses of amboceptor and complement. Indeed very large units of the former were necessary to produce the effect. This influence of titre is clearly brought out by the following experiments:

Exp. I. Haemolytic amboceptor titre, 1–1000. Stock dilution for the purpose of experiment, 1–40. Amount of complement, 0·1 c.c.

The following series were put up:

Table IX.

Showing the effect of titre on the appearance of inhibition.

Tube	Quantity of amboceptor (1–40)	R.B.C.	Complement	Result
	c.c.		c.c.	
1	0·35	0·05 c.c. of a 20 % suspension of homologous corpuscles in each	0·1	Haemolysis in all the tubes
2	0·3		,,	
3	0·25		,,	
4	0·2		,,	
5	0·15		,,	
6	0·1		,,	
7	0·05		,,	
8	0·025		,,	

No Neisser-Wechsberg phenomenon was *observed.*

Exp. II. The same experiment repeated again, but instead of stock dilution, undiluted serum was used in the following quantities:

Table X.

Tube	Quantity of amboceptor and dilution		R.B.C.	Complement	Result
		c.c.		c.c.	
1	Undiluted	0·35	0·05 c.c. of a 20 % suspension of homologous corpuscles in each	0·1	Haemolysis
2	1–10	0·35		,,	,,
3	1–100	0·35		,,	,,
4	1–1000	0·35		,,	Partial haemolysis
5	1–10,000	0·35		,,	Slight ,,
6	1–100,000	0·35		,,	No haemolysis

Exp. III. Amboceptor of a higher titre (1–2000) was used.

Table XI.

Showing a weak "inhibition."

Tube	Quantity of amboceptor and dilution		R.B.C.	Complement	Result
		c.c.		c.c.	
1	Undiluted	0·35	0·05 c.c. of a 20 % suspension of homologous corpuscles in each	0·05	Partial haemolysis
2	,,	0·2		,,	,, ,,
3	,,	0·1		,,	Complete ,,
4	1–10	0·35		,,	,, ,,
5	1–100	0·35		,,	,, ,,
6	1–1000	0·35		,,	,, ,,
7	1–10,000	0·35		,,	Almost complete haemolysis
8	1–100,000	0·35		,,	Partial haemolysis

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It will be noticed that the first two tubes show a sort of Neisser-Wechsberg effect. However, complete inhibition of haemolysis was not brought about. The experiment however suggests that if we could only obtain a serum of a still higher titre, the effect might be shown clearer. As it was not possible to obtain such a serum, it was decided to observe what happens, if to different dilutions of the serum corpuscles were added after sensitising them in suitable dilutions of the same serum.

Exp. IV. In eight separate tubes 0·05 c.c. (20 per cent. susp.) of R.B.C. was sensitised with 0·2 c.c. of 1–10 dilution of haemolytic serum. After incubation for 15′ in a water bath at 37° C. corpuscles were centrifugalised. Two series of tubes, A and B, are taken. In the respective tubes in each series varying amounts of amboceptors were added. To each of the tubes in series A 0·05 c.c. of normal corpuscles was added, and to each tube in series B the same amount of corpuscles from the previously prepared tubes (*i.e.* sensitised ones). Tubes in both series were then incubated and a suitable dose of complement added subsequently. Total volume was made up to 0·5 c.c. in each case.

The results are recorded in the following table:

Table XII.

Showing the occurrence of "inhibition" after the addition of sensitised corpuscles.

Tube	Quantity of amboceptor and dilution		Complement	R.B.C. not previously sensitised. Series A	R.B.C. previously sensitised. Series B	Result A	Result B
		c.c.	c.c.				
1	Undiluted	0·35	0·05	0·05 c.c. of a 20 % suspension of homologous corpuscles in each	0·05 c.c. sensitised as described	ǂ	–
2	,,	0·2	,,			ǂ	–
3	,,	0·1	,,			ǂ	–
4	1–10	0·35	,,			ǂ	–
5	1–100	0·35	,,			ǂ	±
6	1–1000	0·35	,,			ǂ	ǂ
7	1–10,000	0·35	,,			ǂ	ǂ

ǂ = haemolysis. – = no haemolysis. ± = partial haemolysis.

It is seen that there is complete haemolysis in all the tubes in series A, while the tubes in series B show a distinct Neisser-Wechsberg phenomenon.

It can therefore be fairly concluded that inhibition could be observed with those sera which possess a high haemolytic titre.

In all the foregoing experiments after standardisation of complement, that quantity of complement was used which gave quite definite haemolysis very readily. The dose employed therefore was in all cases more than one M.H.D. If only one M.H.D. is used, or as near it as is technically possible, and the test made more delicate, inhibition is more readily observed.

Exp. Two tubes—A and B—are taken. In A 0·1 c.c. of 1–2 dilution of haemolytic serum is placed, and in B, the same amount of 1–300 dilution. To both 0·05 c.c. of 20 per cent. suspension of R.B.C. was added. They were

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then kept in a water bath for 15′. 0·033 c.c. (1 M.H.D.) of complement was added to both.

Result. Only partial haemolysis in tube A, with a marked deposit of red cells at the bottom after centrifugalising. Tube B showed complete haemolysis. This was therefore a clear Neisser-Wechsberg phenomenon.

In tubes showing inhibition it was easy to demonstrate the presence of free amboceptors in the supernatant fluid, after centrifugalisation. The following experiment was carried out:

Exp. For this purpose a number of tubes, like the "A" tubes in the last experiment, were put up. They showed the Neisser-Wechsberg phenomenon. After centrifugalising, the supernatant fluids from different tubes were added together and the mixture was then used as a haemolytic serum. An experiment was made on the lines previously indicated, the results being as follows:

Table XIII.

Showing the presence of free amboceptors in the inhibiting fluids.

Tube	Haemolytic fluid in quantity c.c.	R.B.C.	Complement c.c.	Result
1	0·3	0·05 c.c. of a 20 % suspension of homologous corpuscles in each	0·033	Haemolysis
2	0·1		,,	,,
3	0·05		,,	,,
4	0·025		,,	,,
5	0·0125		,,	Partial haemolysis
6	0·0063		,,	No haemolysis

It is seen here that 0·025 c.c. of inhibiting fluid was able to produce partial haemolysis, higher quantities showing a complete one. There is thus clear evidence of the existence of free amboceptors in the inhibiting fluid which effected the sensitisation of the newly added erythrocytes. A consideration of the haemolytic titre of such an inhibiting fluid shows that it hardly reaches 1–100, the titre of the original serum being more than 1–1000. It is thus seen how great an amount of amboceptors can be taken up by corpuscles—an amount far in excess of that actually necessary to bring about their lysis.

It may be pointed out here in passing, that during the Wassermann reaction as ordinarily done, there is no likelihood of encountering such an accidental Neisser-Wechsberg phenomenon. Sensitisation of corpuscles is brought about by the employment of weak doses of immune serum, and the dose of complement employed is usually $2\frac{1}{2}$–3 M.H.D. The two conditions, therefore, which induce inhibition—enormous excess of amboceptors, and only one M.H.D. of complement—do not exist.

DISCUSSION.

Having thus outlined the conditions under which inhibition of their specific action could be observed, both with haemolytic and bacteriolytic sera when employed in large doses, it becomes necessary to inquire into the causa-

tion of this paradoxical result. It was shown in the historical introduction that attempts had been made to attribute this phenomenon of inhibition, either to the presence of known antibodies such as bacteriolysins (Neisser and Wechsberg), agglutinins, or precipitins (Gay), or to antibodies of a special nature—"inhibiting antibodies"—produced simultaneously with others, during immunisation, which when present in sufficient quantities produced the result (Grüber, Levaditi, Thjøtta). It is therefore necessary to examine these hypotheses in detail.

Firstly, let us take the explanation of Neisser and Wechsberg. Their reasoning (which has already been alluded to) could only be accepted if it could be definitely proved that complement could unite with the amboceptor alone, and not necessarily with the antigen-amboceptor complex. Reasons brought forward in support of this theory, however, no longer hold. Ehrlich and Sachs (1902), for instance, have recorded an instance where guinea-pig's corpuscles were rapidly haemolysed by fresh horse serum when acting simultaneously with inactive *normal* ox serum. Not only had fresh horse serum and inactive normal ox serum singly no such effect, but even if suspended first in inactivated ox serum, *i.e.* submitted to the action of natural amboceptors, and then exposed to fresh serum after centrifugalisation no haemolysis of the corpuscles was observed. They therefore held that normal amboceptors present in ox serum united first with horse serum complement, and then with the corpuscles to bring about their lysis later. That this explanation is not necessarily true has been shown by Bordet and Gay (1906), who subsequently studied the phenomenon thoroughly. They came to the conclusion that bovine serum contains another substance, "bovine colloid" (afterwards named "conglutinin"), which possesses the property of uniting with the cells laden with amboceptors and complement and thus assists in their action.

It is true the ability of the amboceptor to unite with the complement alone has not been definitely disproved in spite of the evidence recorded above; but facts brought forward in the present investigation do not support the hypothesis. Thus no definite relation between bactericidal and inhibitory titres was seen; and during immunisation when bactericidal titre is increased, indicating the production of enormous numbers of amboceptors, inhibition tends to disappear. Thjøtta also observed that in certain immune sera no inhibition was observed at all though they possessed high bactericidal titres. During some experiments with haemolytic sera it was noted that inhibition could only be observed when the serum was of a high titre—thus indicating the possibility of amboceptors taking part in the action; and that they acted differently by not absorbing complement, as will be shown later. Therefore the explanation of Neisser and Wechsberg cannot be accepted.

A question now has to be answered: Do precipitins and agglutinins interfere with bacteriolytic and haemolytic action under certain conditions and produce the phenomenon of inhibition? Before this question is discussed it must be mentioned at the outset that the so-called absence of specific action in large

doses is met with not only with bacteriolysins and haemolysins, but the phenomena of precipitation and agglutination present similar problems as well. The occurrence of a "Zone of No reaction" with agglutinating sera is well known. Though I could not definitely observe the inhibition zones with either bacteriolytic or haemolytic sera, nevertheless, I obtained many indications during my experiments of the possibility of their existence. It is curious that though I was unable to produce the phenomenon at will, whenever I observed it, it was almost always with 0·1 c.c. or 0·05 c.c. of the dose of my immune sera.

That agglutination has no direct action in preventing either haemolysis or bacteriolysis has been definitely proved by Lipstein, whose work has already been referred to in the introduction. Agglutination may of course impede haemolysis. As has been observed by Ehrlich and commonly experienced by many, in haemolytic experiments when corpuscles become strongly agglutinated, frequent mechanical shaking is necessary to bring about their lysis. It however never inhibits haemolysis. In the course of his experiments Sormani had found, and I also obtained similar results occasionally, that the degree of agglutination did not always correspond with the clear appearance of the Neisser-Wechsberg phenomenon. He observed no inhibiting action, when there was first strong agglutination, and when inhibition was distinct, agglutination was either weak or moderate in degree. (Sormani, 1916, see Table IV in the art.)

The analogy between inhibition as met with in bacteriolysis and as seen with precipitating sera is still more striking. The following protocol (taken from Zinsser, 1918, p. 263) will show this clearly:

Table XIV.

(From Zinsser.)

Sheep serum, 0·5 c.c.	Anti-sheep serum from rabbit	Precipitate
	c.c.	
Dilution 1–10	0·5	–
,, 1–50	,,	±
,, 1–100	,,	+ +
,, 1–500	,,	+ + +
,, 1–1000	,,	+ +
,, 1–5000	,,	+

Precipitate more marked with 1–500 dilution, while 1–10 dilution gave no precipitate at all.

Gay has shown that such a precipitate has the property of fixing complement, and has advanced that hypothesis to account for lack of complement action in haemolysis. Sormani's experimental work in refuting these assertions has already been reviewed. Similar experiments, carried out by me with bacteriolytic sera, fail to confirm Gay's hypothesis. The following experiment may be taken in illustration:

Exp. Cholera vibrios from two agar slopes were emulsified in sterile normal

saline solution and shaken in the shaking machine for 24 hours. This is called the "precipitating solution."

To different quantities of amboceptors in each tube, the usual dose of cholera vibrios was added, and in addition 0·1 c.c. of the precipitating mixture. 0·05 c.c. of fresh serum was added as complement, and the total volume made up to 0·5 c.c. with saline solution.

A similar series was put up, with the same doses of antigen, amboceptor, and complement, but without the precipitating solution. These served as a control.

The following table gives the result:

Table XV.

Showing the result on addition of a specific precipitin.

Tube	Amboceptor quantity	Antigen	Precipitating solution	Complement	Result: 1st series, A	Result: 2nd series, without precipitating solution, B
	c.c.		c.c.	c.c.		
1	0·2	2 mm.	0·1	0·05	Many	Many
2	0·1	loop of	,,	,,	,,	,,
3	0·05	48 hrs	,,	,,	0	0
4	0·025	culture	,,	,,	0	0
5	0·0125	in each	,,	,,	0	0
6	Control as usual					

As will be seen from the table, addition of an excess of precipitating solution made no difference in the appearance of inhibition. If the formation of the precipitate had anything to do with it, all the tubes in series A ought to have shown complete absence of bactericidal action.

It may perhaps be argued that the production of a visible precipitate is not absolutely necessary to fix a part of the complement but invisible precipitates formed from minute quantities of precipitonogen have also the same property, as the following table from Muir and Martin's (1906) paper shows:

Table XVI.

Fixation of complement by precipitates (Muir and Martin).

Antiserum	Precipitonogen G.P. serum 55°	Complement c.c. 0·05	0·1	0·2	0·3	0·4
c.c.	c.c.					
0·025	0·1	$\frac{1}{3}$	Complete	Complete	All complete	
,,	0·01	0	0	Complete		
,,	0·001	0	0	$\frac{1}{4}$ haemolysis		
,,	0·0001	0	Almost complete	Complete		
,,	0·00001	Almost complete	Complete	Complete		

It will be seen from this experiment that 0·001 c.c. of serum or precipitonogen has the property of fixing complement to a great extent. (Incidentally this also shows an inhibiting action with large doses!)

These conditions however do not operate during experiments with bacteriolytic or haemolytic sera. For in them the amount of precipitonogen, *e.g.*

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bacilli, is always constant and it is the precipitin (*i.e.* the immune serum) which is added in increasing quantities. That this distinction is an important one is shown from the following observation also taken from their paper:

"We may vary the conditions of experiment by keeping the amount of serum fixed and varying the amount of antiserum. In this case Moreshi found, as shown in his table No. 3, that on increasing the amount of the latter, an optimum point was reached, beyond which additional increase of antiserum resulted in *the diminution in the amount of complement taken up*. We have made a large number of observations with varying results[1]."

It is thus clear that fixation of complement by specific precipitates cannot be admitted as a correct explanation of inhibition, for the above observations show that with higher doses of immune serum no such fixation of complement (at any rate invariably) takes place, while it is with higher doses that we get the phenomenon of inhibition almost invariably.

Gay advises washing the corpuscles repeatedly, five times at least, to remove the last trace of their native serum. In my experiments they were often washed as many as six times. Yet it is possible to free them from serum in another way. If the corpuscles are first sensitised in a weak dilution of the homologous antiserum, and then centrifugalised, they cannot carry even a trace of their original serum, which having formed a precipitate with the antiserum must have been removed when the supernatant fluid was thrown away after centrifugalisation. Yet such sensitised corpuscles themselves show the phenomena of inhibition better, as will be seen from one of the experiments recorded previously (see Exp. IV, p. 417).

From these considerations it must be concluded that neither agglutination nor precipitation adequately accounts for the phenomenon under consideration; and as the inhibiting faculty is a common attribute with all such sera, it is probable that some common factors operate in all cases which produce such paradoxical results beyond a certain dose of the reacting antibody.

As noted previously, inhibition was not observed with fresh active immune serum. Allusion has also been made to Brekke's (1916) work, where he obtained identical results with typhoid sera. This suggests the possibility that "complementoids" produced by inactivation of the serum may interfere with the normal and specific action of bacteriolysins and haemolysins. Ehrlich (1902) has also recorded a case in which normal amboceptors in a dog's serum could be blocked by complementoids, thus rendering them useless for subsequent sensitisation of guinea-pig's cells. Muir and Browning (1906) have also shown that in presence of complementoids, a larger dose of complement is required to bring about the lysis of sensitised cells than is necessary if they were suspended in physiological saline. The following experiments were done to ascertain if complementoids could be implicated in the production of inhibition.

Exp. Two tubes, A and B, each containing 0·025 c.c. of inactivated immune serum (vs. cholera) were prepared. Both were inoculated with one loop of

[1] *Journ. Hygiene*, 1906, VI. 282.

cholera culture and 0·05 c.c. of complement added to each. Total volume was made up to 0·5 c.c. in each tube, in tube A with inactivated normal rabbit serum (therefore containing complementoids) and in tube B with normal saline. Both were incubated for $1\frac{1}{2}$ hours. Both showed complete bacteriolysis after plating, and no inhibition.

The same effect can be shown in yet another way. Complement can be removed either by heating or by bringing the serum into contact with a substance for which complement has a combining affinity. Using the latter method, where no complementoids could be present, it was also possible to observe the phenomenon of inhibition. The following experiment illustrates this:

Exp. To 0·4 c.c. of an *active* immune serum, *an excess* of previously sensitised corpuscles was added, and the tube incubated for $\frac{1}{2}$ hour. Haemolysis took place. The serum was removed from the excess of corpuscles by centrifugalisation. As a precautionary measure the serum was heated to 55° C. to prevent any surplus complement being left over. Here there were no complementoids as the complement was previously used up for haemolysis. To such an immune serum, the usual loop of cholera culture and 0·05 c.c. of complement were added, the total volume being made up to 0·5 c.c. with saline.

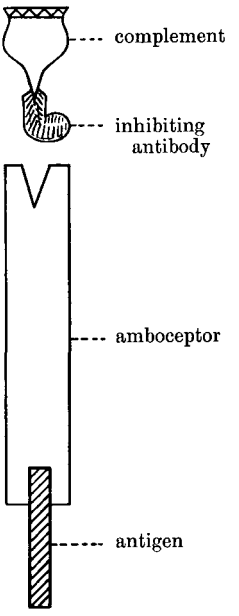
Result. No bacteriolysis took place, plate culture showing enormous number of colonies.

In view of these findings, the theory that complementoids interfere with bacteriolysis to produce inhibition, cannot be accepted. It is also possible, as will be shown later, to explain the findings of Muir and Browning by a totally different hypothesis.

Lastly, the point that inhibition is due to special inhibiting antibodies, has to be considered. Before Thjøtta such an explanation was also offered by Grüber (1901), who believed that anti-alexines were developed during immunisation which acted anti-bactericidally, and anti-haemolytically as well; anti-alexines, therefore, according to him, were not specific. This is in contradiction to what is usually seen during the immunising process, and Lipstein has characterised his statement "as an assumption made solely for the purpose of furnishing an explanation for the phenomena, not based on Ehrlich's views."

Thjøtta's antibodies are however entirely specific in that they require the active co-operation of the specific antigen. The nature and mode of action of these antibodies have been briefly alluded to previously. Arguing wholly in the light of Ehrlich's side-chain theory he first conceives the existence of an inhibiting antibody, and then disproves the possibilities of its attaching itself anywhere in the antigen-amboceptor complex—either between the antigen and the amboceptor, or at either end of the formed complex—in a way which would inhibit the subsequent action of complement. As he did not find, however, any free complement in mixtures showing inhibition, he suggests its union with the complement itself, thus establishing its anticomplementary

nature. The following diagram taken from his paper explains his conception about its action. When such antibodies are present in excess or in sufficient quantities which can only occur when large quantities of serum are used, all or many of the complement molecules are rendered functionally useless and inhibition is brought about.



From Thjøtta, *Journ. Immunology*, Vol. v, 1920.

Many theoretical and experimental objections could be raised to such a conception. In the first place: how is one to account for the origin of such antibodies? It is difficult to understand how one and the same antigen produces, in addition to an enormous number of amboceptors, another body in small amount but equally specific, and yet acting on a complement alone, which is not specific.

His explanation also appears rather confusing[1]. He apparently conceives two distinct antibodies, one precipitate-producing, the other fixing the complement—a conception for which there is no experimental justification. The idea was perhaps necessary to meet Sormani's experimental results recorded before. Moreshi, to whose work the author refers for similarity in explanation, does not indicate such a duplicate nature of the precipitating antibodies. The precipitate alone fixes the complement—it may be either visible, or in so minute an amount as to be invisible.

If, however, the theory is correct it must comply with the following conditions. Firstly, if from a certain quantity of serum amboceptors are removed by absorption with homologous antigen, and the latter removed by centrifugalisation, the supernatant fluid, if subsequently added in a sufficient quantity to a non-inhibiting mixture of antigen-amboceptor and complement, ought to produce inhibition in the latter. Absorption will only remove the homologous amboceptors; inhibiting antibodies, which will not be so absorbed, will still remain free in the fluid. The following experiment was done to test this possibility.

Exp. 0·4 c.c. of immune serum with 0·4 c.c. of saline were absorbed after the addition of one agar slope of living cholera bacilli. The mixture was incubated overnight, and centrifugalised next morning. The supernatant fluid is called the inhibiting fluid.

The following series was then put up:

Tube I. 0·05 immune serum.
+ 2 mm. loop cholera vibrios.
+ 0·05 complement.

[1] *Journ. Immunology*, 1920, No. 1, p. 35.

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Tube II. 0·05 immune serum.
+ 2 mm. loop cholera vibrios.
+ 0·05 complement, and also
+ 0·2 c.c. of inhibiting fluid.

Tube III. 0·025 of immune serum.
The rest as above.

Total volume, 0·5 c.c. in each; mixture plated after the usual incubation for an hour and a half.

Result. All the three tubes showed complete bacteriolysis, and no inhibition at all. 0·2 c.c. of inhibiting fluid corresponded with the hypothetical antibody contained in 0·2 c.c. of unabsorbed serum which always gave inhibition. Tube No. I having no inhibiting fluid, acted as a control.

Experiments were repeated with still larger quantities of the inhibiting fluid with identical results.

Exp. A similar experiment was performed with 0·05 c.c. of normal active serum (quantity in itself bactericidal) and 0·3 c.c. of inhibiting fluid.

Result. Complete bacteriolysis. No inhibition.

As these experiments show, the first condition cannot be satisfied. Secondly, it must be possible to remove the inhibiting antibody from the serum, by allowing it an opportunity to combine with the original complement, in presence of a specific antigen. Such a treated serum, if it is next inactivated by heat to remove the remaining complement molecules, and the usual bactericidal test performed with it with foreign complement, ought to show bactericidal effect, and no inhibition. The following experiment was done to verify this possibility:

Exp. To 0·4 c.c. of active immune serum in a tube 6 loopfuls of dead cholera vibrios were added. After incubation, the serum was inactivated. The inhibiting antibodies, if there be any, were thus rendered functionally useless. The usual dose of cholera vibrios (living) was added subsequently with 0·05 c.c. of complement, total volume being made up to 0·5 c.c. with saline solution.

Result. Inhibition is still observed.

Exp. Above experiments repeated, but instead of dead vibrios 0·2 c.c. of precipitating solution (see p. 421), *i.e.* antigenic solution, was added. This provided sufficient antigen for the inhibiting antibodies to combine with the complement. Subsequent steps as above.

Result. Inhibition.

These experiments show that inhibiting antibodies are unable to unite with the native complement. This becomes yet more inexplicable when it is recalled that nothing prevents them from uniting with it when the same serum is added as complement to the inactivated one (see p. 413).

The results of these experiments do not justify Thjøtta's conceptions, and as the necessary conditions previously laid down for the acceptance of his hypothesis are not satisfied, his explanation must be considered unsatisfactory.

What then is the probable explanation of this paradoxical phenomenon? In mixtures showing inhibition there is no doubt that the antigen-amboceptor-complement complex is somehow disturbed. Something happens which prevents the effective action of complement. Where, and what is the nature of this disturbance? A review of the work of the previous experimenters has shown that they chiefly focussed their attention on the behaviour of complement. They failed to find the complement free in the fluid in tubes showing inhibition, and they concluded that it united with some other antibodies—complement having a greater affinity for them than for the usual amboceptors. Such is the genesis of those various theories which have been already discussed.

There are, however, other possibilities to be considered in this direction. Instead of directing one's attention solely to the complement it is also possible to imagine that something happens to the bacteria or to the cells which enables them to escape the action of complement altogether. Sormani had indeed considered this possibility. His experimental work has been reviewed already. His conclusions however cannot be accepted. It will be recalled that he attributed the protective action in large doses of immune serum to the alteration in the surface of the antigen. Yet, as he himself says, the alteration of surface brought about by a still larger dose produced haemolysis without even the assistance of complement. It cannot be reasonable therefore to attribute these diametrically opposite functions to one and the same property. Besides, as Thjøtta has shown, the bacilli exposed to inhibiting mixtures are nevertheless as susceptible to the action of normal serum, as those which were not so treated. Contact with inhibiting serum afforded them no degree of protection.

With regard to the production of "specific fragility" (as Sormani names the alteration in the surface of the antigen) my experience is somewhat interesting. I suspended 0·1 c.c. of a 25 per cent. suspension of washed sheep cells in 0·5 c.c. of undiluted specific haemolytic serum after incubation for 15′ in a water bath at 37° C. and found that the cells were completely agglutinated. But only a gentle shaking brought about their haemolysis immediately. In order to find out if it was a serum effect or an effect due to amboceptor action the same quantity of red blood cells was suspended in normal horse serum. Here too haemolysis was obtained! Similar suspensions in normal rabbit serum, or in rabbit serum immunised against cholera, had no such effect. Both the above samples of specific and normal horse serum were obtained from Messrs Burroughs Wellcome & Co. It is known that they add a small amount of tri-kresol to them as an antiseptic. The question was if the antiseptic had anything to do with it. I therefore obtained from them a serum (specific against sheep cells) without any antiseptic, and having a fairly high titre. Contact with such a serum for even 24 hours produced not a trace of haemolysis but of course strong agglutination. Similarly dried haemolysin was dissolved in normal saline solution. Contact in such a solution

had no such effect. Further, when a small amount (0·3 per cent.) of tri-kresol was added to the antiseptic-free serum, and the experiments repeated with it, haemolysis without the co-operation of complement was again observed. It had therefore to be concluded that the fragility of corpuscles observed in such experiments was due to the amount of tri-kresol present in the serum. It may here be pointed out that Sormani too had used the serum obtained from proprietary firms; whether his results were also due to the presence of an antiseptic one of course cannot say, although it appears probable.

If then bacteria or cells are not thus rendered invulnerable to the action of complement, there only remains one possibility to be considered. It may happen that, though once united to the amboceptors, bacteria or cells somehow or other extricate themselves from their attachment with them, and lie free in the suspending fluid. Not being sensitised as before, complement will be unable to exert its lytic action alone. There is nothing to prevent their subsequent growth and they therefore actively multiply. I believe this is what happens when we get inhibition.

That such a dissociation of antigen-antibody complexes does take place under the influence of chemicals was, of course, long known. It can also occur spontaneously. It has been particularly observed and studied with agglutinins. Morgenroth (1903) quotes an experiment of Landsteiner, who succeeded in liberating the agglutinin, from agglutinated red blood corpuscles, by digestion at 50° C. with physiological salt solution. He also found that some agglutinin was liberated at even lower temperatures. These observations have been confirmed by other workers also. Jervel (1921), who has particularly studied this aspect of the question, came to the conclusion that red blood corpuscles show more pronounced agglutination at low than at high temperatures, and agglutinin absorbed at a low temperature (8° C.) is again lost when corpuscles are placed at a higher temperature (above 25° C.).

With regard to dissociation of haemolytic amboceptors the experiments of Morgenroth (1903) are particularly interesting and tend to throw some light on the same phenomenon as I suppose is met with in "inhibition." It is known that blood cells (or any cells which act as antigens) are capable of taking up an enormously large amount of amboceptors, far more in excess than is actually necessary for their adequate sensitisation. It was while investigating the firmness of union in such cases that Morgenroth made his curious observation. After sensitising the cells very heavily, as indicated, he tried to obtain them, by repeated centrifuging and resuspension in salt solution, in a medium entirely free from "recognisable amboceptors." Another lot of blood cells of the same species, but unsensitised, was added to this suspension. After a suitable dose of complement was added it was found that the entire amount of blood cells was completely haemolysed. During the incubation of the mixture, therefore, the heavily sensitised cells parted with some of the amboceptors, which were then taken up by the newly-added cells. After this redistribution of amboceptors the complement was easily able to

haemolyse the entire mass. No such transference of sensitiser could take place, however, once the complete haemolytic complex (*i.e.* the antigen-amboceptor-complement union) was formed.

How can one then attribute the inhibition of bactericidal or haemolytic function to such a dissociation of the antigen-amboceptor complex? Inhibition is always observed with very large doses of immune sera—very large indeed with the haemolytic ones. The primary factor in dissociation—the presence of excess of sensitiser—therefore definitely exists. We cannot accept dissociation as a true explanation of the Neisser and Wechsberg phenomenon however, unless the following conditions are completely satisfied.

I. It must, for instance, be definitely shown that red blood corpuscles from the tubes showing inhibition have lost their amboceptors, and being thus entirely "desensitised" are no longer amenable to the action of complement.

II. If the incubation time before the addition of complement is prolonged, inhibition ought to be more marked, for in that case corpuscles would have sufficient time and greater opportunity to withdraw themselves from their union with such amboceptors.

III. Dissociation will not take place once the complement is added, and as it is supposed to take place before, complement added subsequently, not being able to unite with any complex, ought to be found free in the fluid.

An attempt will be made presently to see if experimental proofs could be brought forward to satisfy adequately these essential conditions.

To test the first possibility the following experiment was done:

Exp. Five different tubes were taken. Each contained 0·05 c.c. of amboceptor dilution (1–40) and 0·05 of washed cells (20 per cent. suspension). Tubes were incubated for 10′, and after centrifugalising, the supernatant fluid was removed from the sensitised corpuscles. Varying amounts of haemolytic serum were added to these tubes in series and the total volume made up to 0·5 c.c. in all cases with saline. After incubation for 30′ 0·05 c.c. of complement was added.

The result is shown in the next table:

Table XVII.

Tube	Amboceptor quantity	R.B.C.	Complement	Result
	c.c.		c.c.	
1	0·35 undiluted	0·05 c.c. (of a 20 % susp.) first sensitised as explained	0·05	No haemolysis
2	0·2 ,,		,,	,, ,,
3	0·1 ,,		,,	Partial haemolysis
4	0·35 (1–10)		,,	Almost complete haemolysis
5	0·35 (1–100)		,,	Complete haemolysis

This shows clear Neisser-Wechsberg phenomenon. The corpuscles from Tubes I and II were again centrifugalised and suspended in saline. Further additions of complement brought about no haemolysis. This shows that during their contact in the first suspending medium they lost almost all the amboceptors they possessed before so that subsequent addition of complement had no effect. The first condition is therefore satisfied.

The second possibility was tested in the following manner:

Exp. Two different tubes were taken—one containing 0·1 c.c. of amboceptor dilution (1–2), the other the same quantity of 1–300. 0·05 c.c. of washed corpuscles (20 per cent.) was added and the tubes incubated for 15′. 0·05 c.c. of complement was added subsequently.

Result. Partial haemolysis in the first tube and complete in the second.

An identical series was incubated for two hours instead of 15′ as before, and the same dose of complement was added next. No haemolysis was observed in the first tube, while there was almost complete haemolysis in the second.

Table XVIII.

Showing the effect of incubation for a longer period.

Dilution of amboceptor 0·05 c.c. cells	Result after incubation for 15′ before complement addition	Result after incubation for 2 hours
0·1 c.c. (1–2)	Partial haemolysis	No haemolysis
0·1 c.c. (1–300)	Complete haemolysis	Almost complete haemolysis

It is necessary here to refer to one of Sormani's experiments which was done by him primarily to show that excess of amboceptors had nothing to do with the production of the Neisser and Wechsberg phenomenon.

In different tubes he mixed the same quantity of washed cells, with the same amount of amboceptors, contained in a serum dilution 1–10. Some tubes were incubated for 15′ and the rest for two hours. Another series was treated in the same way, but containing the serum dilution of 1–250 only. To all the tubes in both the series different amounts of complement were added. The following table (No. 3) taken from his paper records the results:

Table XIX.

From Sormani—showing effect of incubation for different periods.

Amboceptor dilution	Reaction time	Complement—Quantity								
		0·1	0·09	0·08	0·07	0·06	0·05	0·04	0·03	0·01
1–10	½ hr	+	+	+	+	+	+	+	0	0
1–10	2 hrs	±	±	±	±	0	0	0	0	0
1–250	½ hr	+	+	+	+	+	+	+	±	0
1–250	2 hrs	+	+	+	+	+	+	+	±	0

Sormani then concludes: "That corpuscles which were under the influence of serum for two hours were less soluble than those which were only so for 15′. This contradicts Neisser and Wechsberg because after 15′ there ought to be more free amboceptors than after two hours."

In my opinion Sormani's experiment rather confirms the hypothesis which I have brought forward. After two hours of incubation the corpuscles lost so much of their amboceptors that, while in the first case 0·03 c.c. of complement was able to produce complete haemolysis, in the next 0·1 c.c. brought about only a partial one. This is entirely in accord with the facts observed in the last experiment. Dissociation, moreover, does not take place unless excess of

sensitiser is present. This explains why Sormani did not obtain any difference in results with different incubation time when the serum used was highly diluted.

The results of these experiments therefore satisfy the second condition laid down.

Next, we have to consider the question of complement. It must be admitted that demonstration of free complement in the inhibiting fluid is a matter of difficulty. Thjøtta made the following experiment: An *active* anti-Shiga serum was treated with homologous bacilli. After centrifugalising, the absorbed serum was tested as to its bactericidal action against another strain, "Dysentery III," against which normal serum had marked bactericidal action. Absorbed serum showed no bactericidal effect. He concluded therefore that complement did not remain free in the fluid when bacilli were in contact with the serum in the "zone of inhibition."

It may be said that this experiment is ill-suited for the purpose for which it is conceived. In the first place I have not found any inhibition with active serum.

Secondly, during absorption of such an active serum, a considerable amount of amboceptors and complement must have been used up. Bactericidal effect therefore may not be possible. How is one to suppose that an absorbed active serum would behave as a normal serum, in a way as if nothing happened to it during the absorption?

I have tried to test for free complement in another way:

Exp. By a previous titration it was found that 0·05 c.c. of rabbit's complement could distinctly haemolyse 0·05 c.c. of sensitised corpuscles. With this dose of complement the usual inhibition experiment was performed with inactive anti-cholera immune serum and cholera vibrios. After incubation for an hour the above quantity of sensitised corpuscles was added to all the tubes. Further incubation showed no haemolysis. That inhibition had occurred was proved when an agar slope, inoculated with the material from the mixture prior to the addition of sensitised corpuscles, showed good growth the next day.

The addition of a second haemolytic system failed to detect the presence of free complement.

There is one fallacy here to be taken into consideration. When it is said there is "inhibition" with a quantity of immune serum, it is generally held that there is a complete absence of the bactericidal effect. It however need not be so. It is just possible that a partial bactericidal effect is occluded by the after-growth of the surviving bacilli, which in consequence give innumerable colonies on the plates. That such an occurrence of a partial bactericidal effect is not unlikely is shown by the gradual diminution in the amount of "inhibition" seen in succeeding tubes in a typical "inhibiting experiment." In the majority of haemolytic experiments showing inhibition, partial haemolysis was observed.

If then some amount of complement is thus always used up to produce a

bactericidal effect however slight and subsequently occluded, complement that is left over may hardly be sufficient to bring about the haemolysis of the sensitised corpuscles subsequently added, as was done in the last experiment. Absence of haemolysis in such cases therefore does not necessarily prove the absence of free complement in the fluid.

That this reasoning is correct is shown by the following experiment:

Exp. Four tubes were prepared each containing 0·4 c.c. of inactive immune serum, 2 mm. loop cholera vibrios (homologous antigen) and 0·05 c.c. of complement, and incubated. These may be called "inhibiting" tubes.

Again eight different tubes were put up, each containing 0·3 c.c. of 1–100 dilution of haemolytic serum, and 0·05 c.c. of 20 per cent. suspension of red blood corpuscles. These were incubated and next centrifugalised. Supernatant fluid was removed from each; each now contained only 0·05 c.c. of sensitised corpuscles. These eight tubes were next divided into two series, A and B, of four tubes each. To all the tubes in series A 0·4 c.c. normal saline was added, and to tubes in series B the same amount of fluid from each of the inhibiting tubes. Varying amounts of complement were added to tubes in series A, and the same amount in the corresponding tubes in series B.

The following table gives the results:

Table XX.

Showing the presence of free complement M.H.D. of complement. 0·04 *gave complete haemolysis of* 0·05 *c.c. corpuscles.* 0·03—*only partial.*

Complement	I	II	III	IV
Quantity	0·04	0·03	0·02	0·01
Series A Cells suspended in saline	Complete haemolysis	Partial haemolysis	Slight haemolysis	No haemolysis
Series B Cells suspended in inhibiting fluid ...	Complete haemolysis	Complete haemolysis	Complete haemolysis	Almost complete haemolysis

I. Plating from inhibiting mixtures prior to the addition of sensitised corpuscles showed inhibition.

II. Rapid appearance of haemolysis in series B.

This experiment clearly shows that in the inhibiting series there was some complement present, which, together with the small amount added, produced complete and quicker haemolysis in tubes in series B than in series A, where no such free complement could be present and which therefore served as a control.

As all the three conditions laid down before are thus completely satisfied, it is reasonable to accept dissociation of complexes as the cause of the Neisser-Wechsberg phenomenon.

Dissociation as an explanation of other paradoxical results.

That dissociation could shed much light on the causes of paradoxical results was first shown by Otto and Sachs (1906) during their study of botulism toxin and its antitoxin. They showed that with a suitable mixture of toxin and its antitoxin, the toxinity for the animal is greater up to a point, the *smaller* the fractional part injected. They discovered that in such cases dissociation of the toxin from its antitoxin was the cause of such results. In the foregoing pages I have attempted to explain similarly the results with bacteriolytic and haemolytic sera. It is also possible perhaps to explain similar paradoxical results obtained by other workers on the same hypothesis.

It is known that dissociation depends on various causes. Huntoon (1921) and W. Georgi (1920) have recently pointed out the importance of the suspending medium, presence of salts and other factors. The latter has particularly studied the occurrence of haemolysis in a salt-poor medium. If sensitised corpuscles were suspended in a solution of glucose (4 per cent.) and presence of salts excluded, no haemolysis could be observed with an adequate dose of complement. Addition of a small amount of sodium chloride, however, restored the normal complement action. In a similar experiment, performed with a bacteriolytic system, I observed more pronounced inhibition with the glucose series than with the saline series, though complete absence of bactericidal function was not met with in the latter case.

In the light of these facts some experiments of Muir and Browning (1906), with regard to the action of complementoids, may be differently interpreted. These observers sensitised ox corpuscles with the homologous immune body and suspended them in different media. Using guinea-pig serum as complement they found that if the medium of suspension was inactivated guinea-pig serum a large amount of complement was required to bring about their lysis. Relative quantities of complement required, reckoned as M.H.D.'s, were as follows:

Medium of suspension	M.H.D. complement
0·085 % saline	1
Ox serum 55° C.	2
G.P. serum 55° C.	5·5
G.P. serum and 0·085 % saline (equal parts)	3
Rabbit serum 55° C.	2

Further experimentation revealed that when sensitised corpuscles were suspended in guinea-pig serum complement was unable to unite with them and it was found free in the fluid.

For the details of these experiments the original paper may be consulted, but facts recorded here show the possibility, at least, of dissociation being the cause of such paradoxical results.

Action of sodium chloride as having an inhibitory influence on dissociation is also seen from the table quoted. Perhaps the presence of an excess of sodium

chloride with lower dilutions of serum in bacteriolytic and haemolytic experiments may also be a contributory factor in preventing dissociation in those dilutions.

Some theoretical considerations.

It only remains to be seen how the acceptance of the dissociation hypothesis would explain some of the important points raised during the present investigation.

(1) It was suggested previously in this paper that such paradoxical inhibitory results were to be observed with the action of all the known antibodies. Dissociation is known to occur with all these antibodies. It is reasonable, therefore, to attribute to it the cause of such paradoxical results.

(2) Inhibition was not observed with fresh active sera. It must be remembered that here complement is always present and in excess. No sooner is an antigen-amboceptor complex formed than complement attaches itself to it to form a complete lysin. There is thus no time left for dissociation and hence the absence of the paradoxical result.

(3) It is obvious no such factors are present with inactivated sera. It is also possible that inactivation by heat may alter molecular concentration of a serum and thus produce conditions which in some way facilitate dissociation. The influence of the medium of suspension has been already discussed.

(4) As dissociation can only take place if amboceptors are sufficiently in excess, it is easy to understand why, during haemolytic experiments, no inhibition was observed when the serum was of a low titre, and how it became manifest when the serum used was of a high titre. It seems that not only homologous but heterologous immune bodies also cause the dissociation of the other if the first is present in excess. In connection with their study of "anti-immune body" Muir and Browning (1906) make the following statement: "...all the anti-immune body is in the first place united with the corpuscles treated with immune body, and the bringing of a large amount of immune bodies (natural) into relation with it, effects the separation of the first in a considerable quantity."

(5) Other points, however, are not so easy of explanation. For instance, if an excess of amboceptors is a necessary factor in the production of dissociation, why does the phenomenon tend gradually to disappear as immunisation proceeds, and when more amboceptors are circulating in the blood? Above all, why should the phenomenon be more easily observed with bacteriolytic than with haemolytic sera?

To these questions it is possible to offer a working hypothesis. The process of immunisation is sometimes compared to warfare between the organism and the infecting agent. Amboceptors constitute the chief fighting units of the body. Perhaps it can be conceived, if we are allowed to carry the simile further, that the amboceptors "newly thrown in" in the early days of battle may not be after all efficient soldiers. They do not know perhaps how to

retain positions. As experience matures they behave as more efficient fighting units, retaining permanently the ground once captured.

In other words, in early days of immunisation many amboceptors produced possess a weak combining affinity with the antigenic cells but later they are replaced by those which are capable of a firm union with them.

If this conception—the production of amboceptors of weak combining affinity—is correct, it is easy to explain why it has been observed that after some time bactericidal sera lose the faculty of producing inhibition even when bactericidal titre is not appreciably diminished. For it is natural to conclude that these weak amboceptors, which are after all the cause of such phenomena, disappear before the rest, *i.e.* the stronger ones. This will also explain the findings of Otto and Sachs, who failed to observe any paradoxical results with their toxin and antitoxin which had been kept for over a year, though a fresh stock of both gave identical results as before.

We close this section with the words of Huntoon, with whom we entirely agree:

"Dissociation of such antigen antibody complexes is not a true reversible reaction. Sensitisation with minimal quantities of amboceptors does not show any dissociation. It only takes place when an excess of serum is used for sensitisation. Amount of antibody remaining attached after such a process is in all cases somewhat more than the minimal sensitising dose. Sensitisation in excess of immune serum results in a graduated variability in the firmness of union of the antigen-antibody complex, and those that are less firmly bound dissociate afterwards[1]."

"*Inhibition*" *as an aid to diagnosis.*

Inhibition is specific. This is not surprising when it is considered that the property is only developed during immunisation, depending, as already has been shown, on the dissociation of the antigen from its specific antibody. Thjøtta has however suggested that this function could be used as a diagnostic aid "in infections with *easily soluble bacilli*." This of course limits its usefulness; but even then it seemed doubtful if the reaction could be considered to have more advantages than other methods already at our disposal. The bactericidal power of serum is certainly more delicate and helpful in this respect than the inhibitory one.

A point here has to be considered. Making use of the inhibiting faculty of a specific immune serum, would it be possible to differentiate between the closely allied strains of an organism? While testing for specificity Thjøtta did a few experiments with different strains of dysentery and other bacilli. Among others he examined four different strains of dysentery (his group II), employing an immune serum produced against one of them. Two of the strains showed identical inhibitory titre, the other two not showing any inhibition at all. (It was not however possible to follow his results clearly, as his tables and the text in some places do not agree.)

[1] *Journ. Immunology*, March 1921, p. 121.

As a number of cholera and closely related strains were available (for these I am indebted to the Lister Institute), it was decided to compare them regarding their "inhibitory" function, with a true cholera strain (Italian), against which an immune serum had already been prepared. The results of these experiments are tabulated in the following table.

The results were not very conclusive. Cholera-like vibrios on the whole showed more inhibition than the true ones, though a few exceptions are also met with. Strain 681 (isolated from a cockroach) behaved like a *true* cholera strain, while strain No. 17 (from a true cholera case in Russia) gave marked inhibition, analogous to a cholera-like strain (cf. 719 in the table).

Between true cholera strains themselves no marked difference, as to inhibition, could be found. Experiments of a similar kind were made with a serum produced against a true cholera strain, No. 6 R.A.M.C. No difference between this and the Italian strain could be noted.

It is not possible to draw final conclusions from these experiments. Though from different sources, the strains examined were comparatively few, and there is always the possibility that they all belonged to one and the same strain. The test also, depending as it does on the counting of organisms after plating, cannot be considered as sufficiently delicate for such an accurate differentiation of closely allied strains. The above facts however show that inhibition cannot be said to have any diagnostic value.

Table XXI.

Inhibiting experiments with different strains of V. cholerae.

		v. cholera						Cholera-like			
		17	21	261	57	307		30	558	681	719
No.	*Quantity of immune serum c.c.	Mild case, Russia	Singapore	Nasik	El Tor	El Tor	Italian	From stools, Montazah	Egypt, stools	From cockroach, Bombay	From water, Bombay
1	0·4	145	50	50	100	100	125	Many	∞	13	Many
2	0·3	94	4	13	60	35	90	,,	∞	3	100
3	0·2	85	5	0	—	18	—	90	∞	0	100
4	0·1	40	3	0	0	17	5	80	∞	0	50
5	0·05	7	0	—	—	—	0	—	—	—	30

* Immune serum against the "Italian" strain.

It must be noted here that Douglas (1921), after examination of a number of strains of V. cholerae from widely different sources, came to the conclusion that there was probably only one serological race of the vibrio.

Inhibition in vivo.

When first the mode of action and especially the therapeutic utility of antisera were being investigated, the experimenters often obtained results which they were unable to explain satisfactorily. Neisser and Wechsberg called particular attention to these unsatisfactory results. R. Pfeiffer (1895), working with cholera vibrios and cholera-immune serum, and Löffler and Abel (1896), working similarly with typhoid and coli bacilli and their homologous antisera, had observed that when guinea-pigs, into which a fatal dose

of the organisms was injected intra-peritoneally, were subsequently treated, some with a moderate dose of the protective serum and others with a still higher one, the animals receiving the former survived, while the latter readily succumbed. Neisser and Wechsberg regarded these results as analogous to those which they had observed *in vitro* with such bactericidal sera, and so offered the same hypothesis to explain the phenomenon, and they came to the conclusion that "It is therefore conceivable that an individual can lose its natural resistance by producing too large an amount of interbody in proportion to the amount of its complement. Such an excess of interbody then would act injuriously rather than helpfully."

In view of the results obtained during the present investigation it is not possible to accept the explanation of Neisser and Wechsberg with regard to the failure of specific action *in vivo* of very high doses of immune sera. It may be pointed out that the experiments of Pfeiffer and others were not done primarily to show such injurious action, and they were done at a time when our knowledge concerning the action of immune sera was very limited. It also seems doubtful if those experiments could be regarded as showing *in vivo* a true Neisser-Wechsberg effect. At any rate they do not appear conclusive. For convenience of description and analysis one of the experiments of Pfeiffer is reproduced in the following table.

Table XXII.

From *Zietschr. f. Hygiene* (1895), xx. 215.

No.	Weight in grms.	Dose of bacilli	Dose of serum	Result	Remarks				
36	220	$\frac{1}{3}$ c.c. of fresh agar slant	1·0 c.c.	Death	Cholera injected 11 a.m.		Temp.	37·6	
					Serum „ 2 p.m.		„	36·9*	
					3 „		„	33·9*	
					4 „		„	31·0*	
					6 „		„	29·0	
37	210	„	0·2 „	Lived	Cholera injected 11 a.m.		Temp.	37·7	
					Serum „ 2 p.m.		„	38·0	Rapid infection in
					2.15 „		„	35·4	course of half an
					5 „		„	32·0	hour
					8 „		„	31·0	
					Temperature slowly kept up.				
38	230	„	0·05 „	„	Cholera injected 11 a.m.		Temp.	38·0	
					Serum „ 2 p.m.		„	39·0	Rapid disappear-
					3 „		„	36·0	ance of cholera
					6 „		„	34·2	vibrios
					9 „		„	33·1	
					Temperature slowly kept up.				
39	200	„	0·01 „	Death	Cholera injected 11 a.m.		Temp.	37·3	Death in the night
					Serum „ 2 p.m.		„	37·0	
					2.30 „		„	34·0	
					3 „		„	31·8	Scanty colonies in
					6 „		„	30·0	the peritoneal exudate

* Rapid appearance of infection. Death, 8 p.m. Purulent exudate microscopically sterile; only isolated colonies in culture.

If this is to be taken as an example of a true Neisser-Wechsberg effect the post-mortem examination of the guinea-pig (No. 36) receiving the largest

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dose ought to have revealed the presence of a large number of living vibrios in the peritoneal cavity; but as will be seen from the notes in the table, the peritoneal exudate was microscopically sterile, and only isolated colonies were found in the culture. However the injurious action of the serum observed in the first guinea-pig can be accounted for in another way. It may also be conceived that after the injection of that quantity of serum the cholera vibrios were subjected to immediate lysis, thus liberating suddenly a large amount of endo-toxin (Pfeiffer) or anaphylotoxin (Friedberger), the absorption of which into the system had the injurious effect. The rapid fall of temperature which occurred only after the injection of serum, the absence of even a preliminary rise as was observed in the other two guinea-pigs which lived, and a rapid death as compared with the last guinea-pig which succumbed late in the night and which had received only a very small dose of immune serum—all point to the same conclusion.

It has already been shown that the cause of the Neisser-Wechsberg phenomenon is the dissociation of the antigen from its union with the amboceptor. Such a dissociation is known to occur *in vivo*, and can be experimentally observed. In this connection an experiment of Coplans[1] (1922) may be described:

Young rabbits inoculated with vaccine lymph show the lesion readily. Coplans took anti-vaccinia rabbit serum and heated a portion of it to 52° C. for 2½ hours. With the heated and unheated portions he put up the following mixtures:

A. *Unheated.*

(1) Immune serum, 4 immune units + Vaccine lymph (1 H.S.D.) ($\frac{1}{100}$ c.c. of calf lymph = 1 human scarification dose)

(2) ,, 2 immune units + do.

(3) ,, 1 immune unit + do.

B. *Heated.*

Similar series as in A.

All the mixtures were incubated for half an hour at 37° C. and then inoculated in as many young rabbits, in the skin on the dorsum as well as in the eye. Unheated immune serum afforded complete protection, while with the heated series the rabbit receiving the largest dose of the immune serum showed typical lesion in the eye, though a delayed one, while the other two remained immune. When the experiment was repeated with an immune serum of a lower titre, similar results were not obtained—all the rabbits being more or less protected.

These experiments are of particular interest inasmuch as they support the conclusions which have already been recorded. Thus the influence of titre, inactivation and incubation is clearly seen.

In this connection a reference may be made to the work of Leclainche and Morel (1901) on the bacillus of malignant oedema. When experiments were

[1] Personal communication.

done on the same lines as Pfeiffer's they obtained similar results, but with the "serum-virus" mixtures they did not observe any harmful effects.

In actual practice high-titre sera are used, which are also seldom inactivated *by heat*. It is doubtful therefore if such dissociation can occur and produce harmful results under conditions which necessitate the administration in disease of large quantities of protective serum.

CONCLUSIONS.

(1) The inhibiting phenomenon of Neisser and Wechsberg, as frequently observed with bacteriolytic sera, can also be shown to occur with haemolytic sera.

(2) This phenomenon develops early during immunisation, when it is very marked; but as immunisation proceeds it tends to disappear.

(3) In sera examined some time after their removal the inhibiting faculty is seen to disappear first, before any marked change is noticed in its bactericidal titre. This can be shown to occur *in vivo* also.

(4) The inhibiting faculty is observed only with inactivated sera; fresh and active sera showing complete bactericidal activity at all times. The effects are not however due to complementoids produced by inactivation.

(5) The phenomenon is not due to the action of any of the known antibodies, or special "inhibiting antibodies"; but it is due probably to dissociation of the antigen-amboceptor complex. Such dissociation only occurs when amboceptors are present in excess. This explains why inhibition occurs only with high doses of an immune serum.

The serum to be used should be without an antiseptic. Commercially prepared antisera usually contain a small amount of it. It is shown that corpuscles sensitised in them undergo haemolysis without the addition of complement (see p. 427).

(6) Since it is known that the union of antigen and amboceptor is not always a firm one, it is suggested that during early days of immunisation amboceptors of a weak combining affinity are produced. These are replaced later by those which are capable of a more permanent union with them (antigens).

(7) The name "complement-blocking" is not an appropriate one, as complement could be shown to exist *free* in the inhibiting fluids. It should rather be spoken of as an "amboceptor-dissociation" effect.

(8) Inhibition is specific. An attempt was made to utilise this fact in differentiation of strains of true cholera but it was found to have no definite diagnostic value.

The writer wishes to express his indebtedness to Prof. Hewlett for suggesting the problem to him and also for his kind guidance and encouragement during the course of the experiments. His thanks are also due to Dr Taylor for his valuable assistance.

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