Studies on immunity / by R.M. in collaboration with C. H. Browning, A. R. Ferguson, and W. B. M. Martin.

STUDIES ON IMMUNITY

ROBERT MUIR

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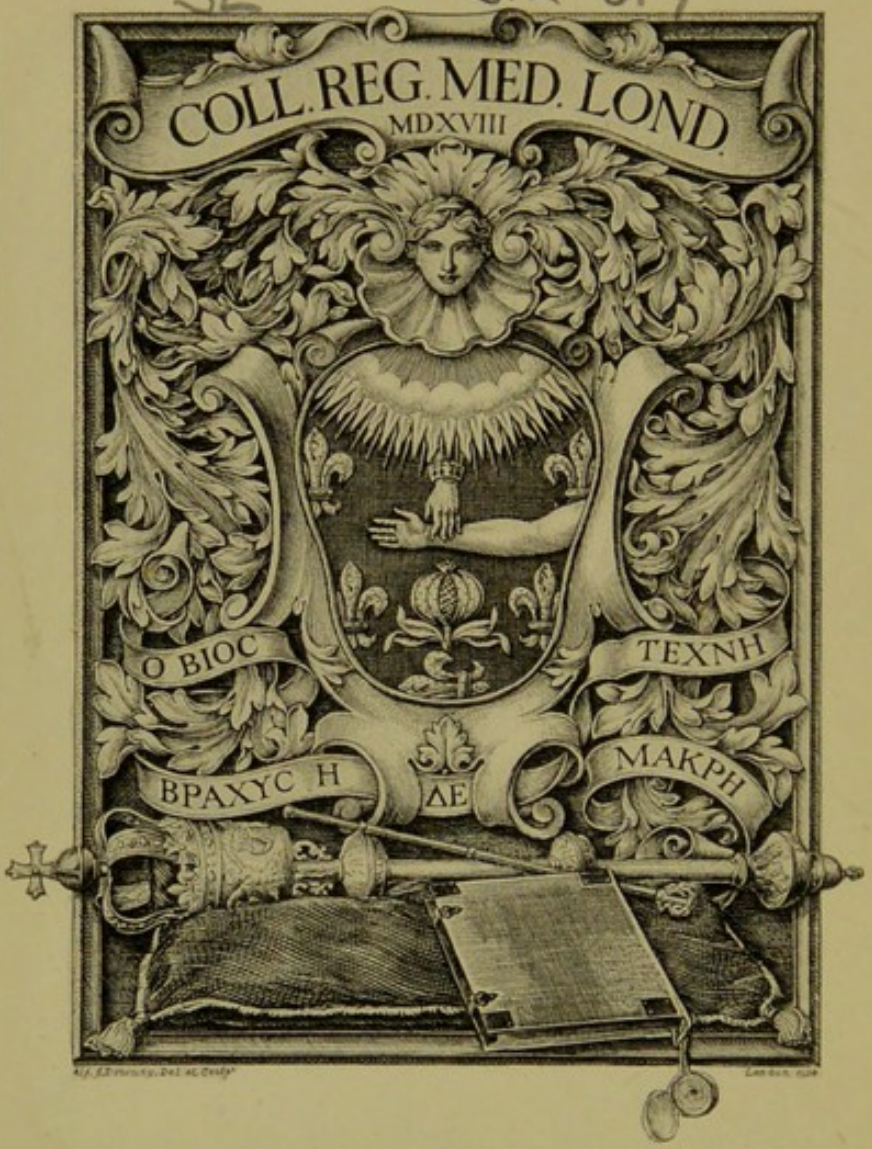

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STUDIES ON IMMUNITY

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PREFACE

THE following pages contain an account of researches on Immunity, carried out in the Pathological Department of Glasgow University and Glasgow Western Infirmary. Practically all the work has been already published, and I have pleasure in expressing my thanks to the Council of the Royal Society and to the Proprietors of the *Lancet*, *British Medical Journal*, *Journal of Hygiene*, and *Journal of Pathology and Bacteriology*, for permission to reproduce the papers in the present form. The volume is essentially a record of original work, and no attempt is made to give a historical review of the whole subject; due reference is, however, given to the results of others, available at the time of first publication, in connexion with the various subjects discussed. The papers have been arranged to give a continuity to the whole, and this arrangement approximately corresponds with the order of publication of the several portions, the only important exception being in the case of the section on 'the Filtration of Serum-Complement', which was the last to appear.

A list of the papers incorporated is appended, and from this the places and times of publication of the several portions of the book will be readily

ascertained. While it has been my endeavour to reproduce the papers as nearly as possible in their original form, certain alterations have been made, the chief of which are the following:—Paper No. 1 has been largely rewritten and extended so as to make it more of the nature of an introduction to the subject. In Paper No. 2 the views formerly accepted with regard to 'Anti-complements' have had to be reconsidered in the light of more recent work on the 'Deviation of Complement'; the changes, however, concern only the interpretation of results. Paper No. 5, which consists essentially of two portions, has been divided; one portion will be found at page 62, the other at page 100. Certain new sections entitled 'Addenda' have been added; for the views expressed in these I am alone responsible. In view of the constant repetition of the same terms in the text, an index has been considered impracticable; in its place a full table of contents has been drawn up.

The greater part of the work has been carried out with the aid of grants from the Carnegie Trustees, and for these I have pleasure in recording my indebtedness. I have also pleasure in thanking Dr. Carl H. Browning for his valuable help both in preparing the work for the press and in the correction of proofs.

ROBERT MUIR.

University of Glasgow
February, 1909.

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LIST OF PAPERS INCORPORATED.

1. R. Muir : ' On the Action of Hæmolytic Sera.'—*Lancet*, 1903, vol. ii, p. 446.
2. R. Muir and C. H. Browning : ' On the Combining Properties of Serum-complements and on Complementoids.'—*Proc. Roy. Soc. London*, 1904, vol. lxxiv, p. 1.
3. R. Muir and C. H. Browning : ' On Chemical Combination and Toxic Action as exemplified in Hæmolytic Sera.'—*Proc. Roy. Soc. London*, 1904, vol. lxxiv, p. 298.
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6. R. Muir and C. H. Browning : ' On the Action of Complement as Agglutinin.'—*Journal of Hygiene*, 1906, vol. vi, p. 20.
7. R. Muir and W. B. M. Martin : ' On the Deviation of Complement by a Serum and its Anti-serum, and its Relations to the Precipitin Test.'—*Journal of Hygiene*, 1906, vol. vi, p. 265.
8. R. Muir and W. B. M. Martin : ' On the Combining Properties of Opsonins of Normal Serum.'—*British Medical Journal*, 1906, vol. ii, p. 1783.
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10. R. Muir and C. H. Browning : ' On the Bactericidal Action of Normal Serum.'—*Journal of Pathology and Bacteriology*, vol. xiii, 1908, p. 76.
11. R. Muir and C. H. Browning : ' On the Filtration of Serum-Complement.'—*Journal of Pathology and Bacteriology*, vol. xiii, 1909, p. 232.

PART I

PROPERTIES OF HÆMOLYTIC SERA

INTRODUCTORY

It is now recognized that in immunity reactions the all-important phenomenon, so far as the serum is concerned, is the appearance of anti-substances which possess a specific combining affinity for the molecules introduced into the animal used for immunization. Such anti-substances may be developed by means of substances in solution, toxins, ferments, proteins, &c., or by means of formed elements, such as tissue cells, bacteria, &c., the specific reaction being brought about by certain of their constituent molecules. The result is, however, essentially the same in the two cases. The molecules which lead to the formation of anti-substances are known as antigens, and it is now generally accepted that they are the same molecules as those for which the anti-substances show the specific affinity, as demonstrated by test-tube or animal experiments. Ehrlich, in accordance with his side-chain theory, has applied the term ' receptors ' to molecules in the cells which have a combining affinity for other molecules, and which, no doubt, functionate in the processes of nutrition. These receptors are the molecules which act as antigens, when tissue cells or bacteria are injected into an animal. Similar molecules may, however, be discharged from the cells and exist free in the body fluids ; thus, for example, we may speak of serum receptors.

The anti-substances developed in immunity reactions are now generally recognized as belonging to three main classes. In the first class, the anti-substance simply combines with

the antigen, filling up as it were its combining affinity, but without producing any other physical change so far as is known. The anti-toxins are the typical members of this class. In the second class, the anti-substance combines with the antigen and also, by a zymotoxic action, produces some recognizable physical change in it, resulting, for example, in precipitation, agglutination, &c. In the third class the anti-substance combines with the antigen without of itself bringing about any recognizable effect in it so far as we know, but it leads to the union of the labile substance in normal serum, generally known as 'complement' or alexine, which often produces some destructive action. The bacteriolytic and hæmolytic sera are the typical examples. It was formerly supposed that anti-substances of the third order of this nature were only produced when formed elements were used for injection, but it is now known that they can also be developed by apparently simple protein substances in solution. From the biochemical point of view the distinctive action of an anti-substance of this order is its leading to the combination of complement.

In the case of a hæmolytic serum we have, therefore, three kinds of molecules concerned: (1) the receptors (R) of the red corpuscles which act as antigens: (2) the corresponding anti-substances developed by immunization and possessing the specific affinity for the antigens, they are usually known as immune-bodies (IB); and (3) the normal constituent of the serum, complement (C), which unites through the medium of the immune-body; it does not increase during the process of immunization. The lysis of the corpuscles by the hæmolytic serum may thus be said to result from the union of R + IB + C. It follows that given any two of these substances, we can test for the presence of the third. This will be abundantly illustrated by the experiments detailed. The relations of the several substances

concerned, owing to the visible effect produced on the corpuscles, can be studied with an exactitude in the case of hæmolysis which is impossible in the case of other sera—hence the importance of the study. The facts ascertained can then be tested in the case of anti-bacterial sera. It may be stated that while numerous striking analogies have been proved to obtain between these two chief kinds of sera, important differences are also brought out. The properties of the three classes of molecules concerned in hæmolysis by an anti-serum will be considered in turn.

Method of Production of Hæmolytic Sera and Estimation of Dosage

The development of a hæmolytic serum is usually a matter of comparative simplicity. It is sufficient to inject quantities of the red corpuscles at suitable intervals. In all cases the corpuscles ought to be washed several times in salt solution, in order to free them from the serum; otherwise serum anti-substances will be developed and a complication may be introduced. Rabbits have been chiefly used in our experiments, and the corpuscles have been injected intraperitoneally. In the case of ox corpuscles, injections of 5, 10, and 15 c.c. respectively, with intervals of ten days between, usually result in the production of a potent serum. The hæmolytic action reaches its maximum from seven to ten days after the last injection. Accordingly, a preliminary test should be made at this time, and if the result is satisfactory, the animal should be killed by bleeding. The blood is collected with aseptic precautions; the serum is allowed to separate, and is conveniently stored in glass tubes, sealed at the ends. It is advisable to expose these to a temperature of 55° C. for an hour on three successive days to ensure sterility. Immune-body is relatively a

stable substance; it may remain practically unchanged in amount for several months when kept in a sterile condition in the dark at ordinary room temperature. The several immune sera are conveniently designated by placing first the name of the animal immunized and then the name of the animal whose corpuscles have been injected; thus a hæmolytic serum stated as *rabbit v. ox* signifies the serum of a rabbit immunised against ox's corpuscles. As von Dungern showed, in the process of immunization the amount of complement does not increase, and, accordingly, the hæmolytic dose of the fresh serum may not represent the richness in immune-body, owing to the lack of sufficient complement. Accordingly, the ordinary procedure is to destroy the complement by heating, as above described, and then to *reactivate* the immune-body by fresh normal serum, say of a guinea-pig or rabbit. A preliminary procedure is to test the hæmolytic dose of the immune-body, which, as is shown below (p. 7), varies with different complements. In the case of the anti-ox serum this is conveniently done with the aid of rabbit's complement (normal serum) as this has no action on the ox's corpuscles. In the case of sera which have some natural lytic action on ox's corpuscles, e.g. guinea-pig's serum, the natural immune-body must of course be removed by treating the serum with ox's corpuscles at 0° C., before the dose of the artificial immune-body can be ascertained. In some instances, however, it is not possible to remove the normal hæmolytic property from a serum by treating it with the corresponding corpuscles at 0° C. (vide p. 72).

A series of tubes is prepared, each containing the test amount of suspension of red corpuscles, namely, 5 per cent. in ·8 per cent. solution of sodium chloride, the corpuscles having been carefully washed to free them of serum. To each tube more than the amount of complement necessary to produce lysis is added, and then to the tubes in series different amounts of immune-body, along with sufficient ·8 per cent.

salt solution to make up to the same bulk in each tube, say 1·5 c.c. A rough test can thus be made, and then a more accurate estimation carried out in a similar way. The dosage of immune-body having been thus obtained, the minimum hæmolytic dose of any complement (fresh normal serum) can be estimated by a similar procedure, in this case an excess of immune-body being added. It is to be noted that if the minimum dose of immune-body and of complement be thus obtained by the above method, in most cases the two minimum doses together will not produce complete lysis. In other words, the minimum dose of immune-body will only produce lysis with the *optimum* amount of complement, which may be several minimum doses, as above defined. A similar statement applies to the minimum dose of complement. The optimum amount of complement and of immune-body, respectively, as compared with the minimum dose, varies considerably in different sera, and it must be determined in each particular case. The explanation of this phenomenon, which is still obscure, has been discussed in a paper by Morgenroth and Sachs.[1] After the addition of immune-body and complement to the red corpuscles, the test-tubes are placed in an incubator at 37° C. for two hours, and are then placed in a cool chamber till next morning, when a reading is taken. Complete lysis is shown by the contents of a tube being quite clear and without any deposit. When subsequent tests or procedures have to be carried out on the same day, the reading may be taken when the tubes are removed from the incubator.

As has already been indicated, the lysis of the red corpuscles indicates the union of immune-body and complement with the receptors of the red corpuscles, but the hæmolytic doses, as above explained, do not indicate how much of these substances may enter into union. On the contrary, as will be shown below, red corpuscles may take

[1] Morgenroth and Sachs, *Berlin. Klin. Woch.*, 1902, no. 35.

up multiple doses of immune-body, and thereafter multiple doses of complement. The lysis in these cases thus represents a *comparatively early stage* in the union of the three bodies concerned. Accordingly, in estimating the possible combining amounts, we must make use of the absorption method, that is, estimate the amount of immune-body and of complement left free after a combining action has occurred. This matter is of great importance in connexion with the experiments detailed below, and supplies one of the most useful methods in the investigation.

In the action of a hæmolytic serum there results some damage to the envelope of the corpuscle, which allows a diffusion of hæmoglobin. The corpuscle is, however, not completely destroyed, as the stromata can still be seen on microscopic examination after lysis. Even when multiple doses of immune-body and of complement are used, the total digestion of the red blood-corpuscle does not take place. Bordet[1] showed that the action of a hæmolytic serum differed from that of ordinary laking agents (water, ether, and the like), inasmuch as the osmotic properties of the corpuscles seemed to be lost in the former case. As is well known, if red corpuscles are laked with water, and then the solution be made hypertonic, the stromata become contracted, just as uninjured corpuscles become crenated, and on diluting again with water they resume their original size. On the contrary, after lysis by a hæmolytic serum, these phenomena are not met with, variations in the salt content of the medium apparently having no effect upon the stromata of the corpuscles. The action of the serum would thus appear to be of the nature of a partial solution of the envelope, leading to the formation of apertures sufficiently large to prevent the osmotic phenomena. Beyond this fact we know practically nothing with regard to the hæmolytic effect.

[1] Bordet, *Annal. de l'Inst. Pasteur*, XIV, 1900, p. 270.

Quantitative Relations of Antigen and Anti-substance. The hæmolytic dose of immune-body is sometimes extraordinarily small. In other words, the amount of immune-body as judged by the proportionate number of molecules appears to be very great. It is now recognized that the amount of anti-substance developed may greatly exceed the combining equivalent of antigen injected. This fact, which is of great importance in connexion with the general question as to the production of anti-substances, is well illustrated in the case of hæmolytic serum. For example, after the injection by the intraperitoneal method of the corpuscles of 30 c.c. of ox's blood, on several occasions a serum was obtained of which ·0005 c.c. was the hæmolytic dose for 1 c.c. of a 5 per cent. suspension of ox corpuscles; that is, ·01 c.c. was the dose for 1 c.c. of undiluted blood, or 1 c.c. hæmolytic serum would dissolve completely 100 c.c. of ox's blood. Taking the total amount of the hæmolytic serum in the animal as 60 c.c., we find that the anti-serum would dissolve 6000 c.c. of the ox's blood. The red corpuscles in question, however, have the faculty of combining with several hæmolytic doses, although it is not possible to say exactly how many. Even when this allowance is made, the anti-serum would satisfy the combining affinities of several hundred cubic centimetres of ox's blood. In other words, the antigens of the corpuscles injected give rise to the production of many times as much anti-substance (immune-body) as would satisfy the combining affinities of the total amount of antigens used.

SECTION A. ON THE PROPERTIES OF IMMUNE-BODIES.

The Specificity of Immune-bodies

This subject is part of the question regarding specificity of anti-substances in general, and the results of investigation correspond with the facts established in the case of precipitins, agglutinins, &c. A hæmolytic serum is always most active towards the corpuscles used in its development, but it may also have some action on the blood of allied species. To give a concrete example: an anti-ox hæmolytic serum with a hæmolytic dose of ·0005 c.c. when reactivated with guinea-pig's complement, was found to produce lysis of sheep's corpuscles in a dose of 0012 c.c. When a small quantity of this serum, however, was treated with excess of sheep's corpuscles, time being allowed for combination, and then the corpuscles were centrifugalized, it was found that the serum had lost its hæmolytic action on sheep's corpuscles, the immune-body having been removed by the previous contact. It still, however, possessed marked hæmolytic action on ox corpuscles, the hæmolytic dose being ·0012 c.c.; that is, the immune-body had been reduced to a little less than half of the original amount. In this instance it therefore appears that about half of the immune-body molecules act equally on sheep and ox corpuscles, whilst the other half act only on ox corpuscles. Or, in other words, half of the hæmolytic receptors of the ox corpuscles are similar (so far as combining affinities are concerned) to those in the sheep, whilst the others are different. Ehrlich and Morgenroth [1] worked out this subject very completely in the case of the corpuscles of the ox and of the goat, the results obtained being similar

[1] Ehrlich and Morgenroth, *Berlin. Klin. Woch.*, 1901, nos. 21, 22.

to those just stated. The apparent non-specificity is thus shown to be really due to the molecules of immune-body being of more than one kind, corresponding to different receptors (antigens) which give rise to them. All the results obtained by absorption methods go to show that when an anti-serum acts on heterologous corpuscles or bacteria, this is due to their possessing some receptors similar to those in the corpuscles or bacteria used for injection. The number of antigens, as tested by their combining affinities, would appear to be practically limitless, and each anti-substance combines only with those similar to the one which has been used in treating the animal.

On the Mode of Union of Immune-body with Red Corpuscles

The union of immune-body with red corpuscles, as Ehrlich and Morgenroth showed in their first communication, takes place at 0° C. This can be readily shown by placing red corpuscles in a mixture of complement and immune-body at 0° C. and, after a time, centrifugalizing and pipetting off the fluid. If the separated corpuscles are then washed in salt solution, and the usual suspension is made, no lysis occurs when they are incubated at 37° C.; whereas lysis occurs at once on the addition of complement. The presence of complement in the fluid which was pipetted off can be of course shown by adding it to sensitized corpuscles, when lysis occurs. A method is thus given for separating immune-body from complement when the two are present in a mixture, and it can be similarly applied in some cases for freeing a normal serum of a natural immune-body when it is present (*vide infra*, p. 28).

The amount of immune-body which may be taken up or absorbed by red corpuscles is conveniently estimated in the following manner. Varying multiples of the minimum hæmo-

lytic dose of immune-body are added to a number of tubes in series: time is allowed for combination, and the tubes are then centrifugalized: the fluid from each is pipetted off and added to the corpuscles of 1 c.c. of suspension (the salt solution having been previously removed by centrifugalization). Sufficient complement for lysis is then added to each tube, and the tubes are incubated at 37° C. for two hours. The quantity of immune-body left free, up to a complete hæmolytic dose, can thus be determined in the case of each tube by the degree of lysis which occurs. The amount of immune-body taken up by the corresponding red corpuscles varies within very wide limits in different cases. Ehrlich and Morgenroth found that in the case of one serum no more than the hæmolytic dose entered into combination; that is, if two hæmolytic doses were added, one remained free in the fluid, whereas in another case a hundred doses had to be added before one dose remained free. In the case of *rabbit v. ox* serum, I have found that the number of doses estimated in this way varies from six to ten; it is usually seven or eight (variations seem to depend both upon the serum in question and upon the corpuscles used in testing).

It is to be noted that the union of immune-body with the hæmolytic receptors of the red corpuscles is not like that of a strong acid and base; that is, there is not a sharp neutralization point. Even when only two doses of immune-body are added, an appreciable amount is found to be left free in the fluid when it is separated, and with more doses this gradually increases until a hæmolytic dose is free, as has already been indicated; but when this stage is reached the red corpuscles are not saturated. If X doses give one free dose, more than X + 1 doses must be added before two free doses are got. This is clearly brought out in the adjoining table, which shows the amounts of free immune-body when varying amounts of immune-body

were added to the standard amount of red corpuscles.

1 free dose of IB was obtained when			12	doses of IB were	added
2 free doses of IB were		,,	16	,,	,,
3	,,	,,	20	,,	,,
4	,,	,,	23	,,	,,

It is accordingly not possible to state exactly how much immune-body can be taken up by the red corpuscles, as the amount entering into combination varies with the total amount present; the condition being, in this respect at least, analogous to mass action. In any case, however, the combining powers may be suitably indicated for comparative purposes by stating the minimum number of doses which will leave one dose free. This, however, is only a conventional standard.

In the combination of immune-body with red corpuscles (anti-substance with antigen) we have an exaggerated example of what is now known as the 'Ehrlich Phenomenon'. In studying the union of toxin and anti-toxin, Ehrlich found that if he ascertained the largest amount of toxin which was just neutralized by a unit of anti-toxin, this amount had to be increased by much more than one lethal dose before the mixture became lethal to the test animal. He explained this result by the complicated constitution of the toxin, and especially by the presence of toxoids which have a weaker affinity for the anti-toxin than the true toxin has. He also considered that the union of toxin and anti-toxin is of firm character; that is, it is not reversible. In the present case, namely, that of immune-body and red corpuscles, it can be readily shown, however, that the union is a comparatively loose one, and that the immune-body can be dissociated. The phenomenon may in this case be therefore readily explained by the reversibility of the combination. This will be discussed in the next section.

On the Dissociation of Immune-body from Red Corpuscles

That the immune-body can be separated from red corpuscles after combination, was shown independently by Morgenroth[1] and myself. The method is to bring red corpuscles, sensitized with multiple doses of immune-body, into contact with fresh corpuscles, and then after a suitable interval of time to add complement and to observe the degree of lysis which occurs. In the original experiments I brought the sensitized and the fresh corpuscles into close contact by means of centrifugalization ; but this is unnecessary, although the passage of the immune-body to the fresh corpuscles takes place more readily than when the corpuscles are simply suspended. The phenomenon can readily be demonstrated in the following manner :—1 c.c. of 5 per cent. suspension of red corpuscles is treated with ten hæmolytic doses of immune-body (rabbit *v.* ox) ; after being allowed to remain at the temperature of the room for an hour, the corpuscles are centrifugalized and the fluid is pipetted off, and then they are washed several times in salt solution and resuspended. All the immune-body is thus in combination with the red corpuscles. Another cubic centimetre of suspension of untreated corpuscles is then added, and the contents of the tube are thoroughly shaken up : the tube is then placed in an incubator at 37° C. for an hour. A sufficient quantity of complement, say four hæmolytic doses, is then added and the tube is returned to the incubator. It will be found that lysis occurs in *all the corpuscles*, thus demonstrating that immune-body has passed from the sensitized to the untreated corpuscles. I have found that in the case of the immune serum, rabbit *v.* ox, the corpuscles treated with twelve hæmolytic doses of immune-body will usually give up two hæmolytic doses

[1] Morgenroth, *München. Med. Wochenschr.*, 1903, no. 2.

to fresh corpuscles after contact for an hour at 37° C.; that is, two cubic centimetres of fresh corpuscles added as in the above experiment will undergo complete hæmolysis on the addition of sufficient complement. Usually one dose of immune-body can be obtained from red corpuscles containing six hæmolytic doses. Dissociation takes place also at the temperature of the room, although much more slowly; while at 0° C. it is practically nil. The separation of immune-body can also be demonstrated without the actual presence of fresh corpuscles. Three tubes are taken, each containing 1 c.c. of suspension of red corpuscles treated with excess, say twelve doses, of immune-body; after an hour at the room temperature the fluid is pipetted off and the corpuscles are repeatedly washed as before; to each tube is added 1 c.c. of ·8 per cent. sodium chloride solution. One tube is placed in an incubator at 37° C.; one is kept at the room temperature; and another is kept at 0° C. At the end of an hour the tubes are centrifugalized and the fluid from each is added to 1 c.c. of untreated corpuscles; sufficient complement is then added to produce lysis. It is found that in the first tube a considerable amount of lysis results; in the second a mere trace; whereas in the third, there is no appreciable lysis. This shows that at 37° C. corpuscles containing multiple doses of immune-body give off a certain amount to the surrounding fluid when this is free of immune-body. In other words, there would appear to be in each case an equilibrium between the combined and the free immune-body which is reached after a certain time. This is one of the most striking, and probably the most easily demonstrable, examples of the dissociation of an anti-substance from its antigen after combination.

If in the experiment above, the sensitized corpuscles are mixed with fresh corpuscles and then complement is added *at once*, lysis takes place only in the sensitized corpuscles, the others remaining almost unaffected. This is due to

the complement becoming fixed to the sensitized corpuscles before there has been time for the immune-body to pass to the fresh corpuscles. The result, of course, also shows that the combination of complement is a firm one, and that dissociation of it does not take place under the conditions mentioned. Morgenroth assumed that the complement locked up the molecules of immune-body in the red corpuscles, but did not experimentally prove that this was the case. The subject will be referred to again below (p. 34).

As the relation of time to the firmness of union of an anti-substance with its antigen has been considered to be of importance, I have performed experiments to determine whether the dissociability of immune-body from red corpuscles becomes less marked in course of time. The amounts of immune-body which can be separated from red corpuscles after one hour and after twenty-four hours have been compared. In the latter case the given number of doses of immune-body are added to the corpuscles and they are allowed to remain at the room temperature for twenty-four hours (it may be noted that the total amount of immune-body capable of combination is taken up in about half an hour). The tubes are then centrifugalized, and the corpuscles are washed in salt solution and re-suspended; the untreated corpuscles are then added and the experiment is carried out as above described. The 1-hour tubes are similarly treated at the same time. The result is that as much immune-body can be obtained by dissociation from the 24-hour tubes as from the 1-hour tubes; that is, there is no evidence of increasing firmness of the union of immune-body in course of time.

The facts with regard to dissociation of immune-body from the corresponding receptors of red corpuscles are of interest in connexion with the results obtained by injecting red corpuscles saturated with immune-body. If the molecules which act as antigens are the same as the receptors

of the red corpuscles with which the immune-body combines, then it is evident that when the combining groups of the receptors are filled with immune-body, such corpuscles should not give rise to immune-body when injected into an animal. Von Dungern[1], who was the first to perform experiments of this kind, found that such was the case—the serum of an animal injected with fully sensitized corpuscles did not come to contain immune-body. Sachs[2] on repeating these experiments obtained in some cases similar results, but in others a certain production of immune-body did occur, though this was small in amount. Neisser and Lubowski[3] obtained similar results to these last by injecting typhoid bacilli saturated with agglutinin, a small amount of agglutinin being formed in the animal treated. From the facts detailed above with regard to dissociation of immune-body from red corpuscles, when they are in a medium free from immune-body, it is evident that within the living body the same may occur, and thus some of the receptors of red corpuscles or bacteria will have their combining group freed, and thus be able to act as antigens. The fact that red corpuscles saturated with immune-body may in some cases give rise to a certain amount of immune-body in the treated animal is thus explained. We cannot say why this should occur in some cases and not in others, though variations in the rapidity of destruction of the corpuscles may play a part.

On the Condition of Immune-body after Hæmolysis

Supposing that red corpuscles containing several hæmolytic doses of immune-body are hæmolysed by the minimum amount of complement, we have to inquire in what state the surplus molecules of immune-body are. There may be

[1] Von Dungern, *München. Med. Wochenschr.*, 1900, no. 20.
[2] Sachs, *Centralbl. f. Bakter.*, xxx, 1901, p. 491.
[3] Neisser and Lubowski, ibid., 1901, p. 483.

said to be three possibilities: (1) the surplus molecules of immune-body may be destroyed in the process of hæmolysis; (2) they may become free in the fluid; or (3) they may remain in union with the receptors of the red corpuscles. It is easy to show in the first place that they are not destroyed, as abundant immune-body can be obtained from the red fluid resulting from the hæmolytic action.

Example: 1. Red corpuscles (say one cubic centimetre suspension) are treated with excess of immune-body, and after suitable time is allowed for combination they are centrifugalized and washed several times in salt solution. 2. Sufficient complement is added to produce complete hæmolysis. 3. One cubic centimetre of untreated corpuscles is then added to the red fluid and the mixture is placed in the incubator for an hour at 37° C. (No hæmolysis occurs because there is no free complement.) 4. The added corpuscles are then separated by centrifugalization and washed in salt solution. On the addition of complement they undergo complete hæmolysis.

As mentioned above, *free* immune-body unites with red corpuscles at 0° C. We may accordingly test whether immune-body is recoverable from the red fluid at this temperature. The steps are the same as in the above experiment, only after hæmolysis has occurred at Stage 2 the tube is placed in a mixture of ice and water, the fresh corpuscles are then added, and the mixture is allowed to remain at 0° C. for an hour. Afterwards the tube is centrifugalized and the corpuscles are washed in cold salt solution. Complement is then added to the corpuscles and the tube is placed in the incubator. The result is that there is practically no lysis, though a control with red corpuscles added to fluid containing a dose of immune-body at 0° C. and then centrifugalized, gives complete hæmolysis on the addition of complement.

We may therefore conclude that when red corpuscles containing several doses of immune-body are hæmolysed by the minimum dose of complement, the surplus molecules of

immune-body do not become free but remain attached to the receptors of the corpuscles ; a certain amount can, however, be dissociated at the higher temperatures on the addition of fresh corpuscles. And it follows as a corollary that in like conditions when immune-body is obtained by adding red corpuscles to a fluid or suspension of corpuscles at 0° C., the immune-body so obtained has been in the free condition.

On the Constitution of Immune-body

As is well known, there are two chief views on this subject, namely, that of Ehrlich and that of Bordet. According to the former, the immune-body has a specific affinity for the receptor of the cell, and another special, though not specific, affinity for the complement ; it acts as a link between the complement and the cell receptor ; that is, is an *amboceptor*. According to Bordet, the immune-body produces some physical or chemical change in certain molecules (the receptors) which brings about a combining affinity for complement, the molecules of the latter then combining directly with the receptors of the cells. The immune-body is a sensitizer, *substance sensibilisatrice*. It is a mistake, however, to suppose that Bordet believes that immune-body inflicts some damage on the cell which makes it susceptible to the entrance of complement ; that is sufficiently evident from his statements with regard to the fixation of complement by albuminoids and their anti-substances. All that is meant in speaking of immune-body as a sensitizer is that, on its combining with the corresponding antigen, complement enters into union with the latter. Accordingly, unless it can be proved that complement combines directly with immune-body, the explanation of the mechanism appears to involve theoretical questions of similar nature according to both views—either the immune-body sensitizes the receptor, leading to the combination of complement, or

the cell receptor sensitizes the immune-body with a corresponding result. Sensitizing thus means only the production of a combining affinity in a molecule which otherwise does not possess it ; and the increase of combining affinity already present in small degree without the presence of an immune-body, is also a part of sensitizing action.

We have thus to consider the evidence for the direct union of complement and immune-body. It may be stated at the outset that this appears to us not to be of conclusive nature. As was first shown by Ehrlich and Morgenroth, immune-body may be separated from a mixture containing complement by adding the homologous red corpuscles at 0° C., the complement not entering into combination at that temperature. Thus immune-body appears to exist separately in the mixture. Ehrlich, however, suggested that the two substances really form a loose union at a higher temperature, say 37° C. Certain recent experiments carried out by Dr. Browning and myself lend, however, no support to such a view. It is shown below (p. 90) that on filtering fresh serum through a Berkefeld filter, complement is in great part retained ; the first few cubic centimetres which pass through contain almost no complement. On the other hand, immune-body passes through the filter practically unaltered. Now, if complement combines with immune-body at 37° C., and if complement is retained by the filter, then on a mixture of the two being filtered at 37° C. the immune-body in combination with complement should also be retained by the filter. We have found, however, that such is not the case in the circumstances mentioned—as much immune-body passes through when mixed with complement as when it is filtered alone. These experiments accordingly lend no support to the view that complement unites directly with immune-body at 37° C.

The Neisser-Wechsberg phenomenon has also been used in support of the amboceptor theory. The essence of this

phenomenon is the fact that when a mixture of immune-body and complement brings about a bactericidal action, this may be annulled by increasing the amount of immune-body. According to the above theory, it is supposed that in such a case free immune-body has as much affinity for complement as immune-body combined with bacteria has. Accordingly, when immune-body is present in excess, many of the molecules of immune-body taken up by the bacteria will have no complement attached to them, and thus bactericidal action will not result; whereas, when the amount of immune-body corresponds to that of complement, each molecule of immune-body combining with a bacterium will bring with it a molecule of complement, and thus a greater bactericidal effect results. This, however, is only a theoretical explanation of the phenomenon, and facts have been brought forward which suggest that it is not the correct one; especially the fact observed by Buxton, namely, that if a particular mixture of complement and excess of immune-body has no bactericidal effect, such effect may be produced on diluting the mixture, a result which appears quite unintelligible according to the above explanation.

Ehrlich[1] states in support of his view, that an immune-body acts best in association with the complement of the animal from which the immune-body has been obtained. He cites the interesting result of Wechsberg, that a heated immune-serum for the v. Metchnikovi when obtained from the rabbit is activated by rabbit's complement, and not by pigeon's; whereas a corresponding immune-body got from the pigeon is activated by pigeon's but not by rabbit's complement. This, however, might well be the case according to the other theory as to the constitution of immune-body, for if an immune-body is a sensitizer it is only natural that it should be adapted to act well in association with the complement

[1] Ehrlich, *Festschrift zum sechzigsten Geburtstage von Robert Koch*, 1903, p. 509.

from the same animal. Furthermore, in speaking of suitability of complement, a distinction has not been drawn between combining affinity and toxic effect. As judged from the minimum hæmolytic dose for ox's corpuscles, guinea-pig's complement acts better with the immune-body from the rabbit than does rabbit's complement itself. This is chiefly due to the fact that the guinea-pig's serum has a greater number of active molecules than the rabbit's has (p. 45), but it really also combines more efficiently as judged by absorption experiments. Too few examples have been intimately studied to permit a definite statement to be made on this subject.

Another argument in favour of the amboceptor view is drawn from the facts established with regard to cobra venom by Kyes[1]. The chief points are, that the hæmolytic toxin of this venom in many cases cannot by itself produce lysis of corpuscles, but requires the addition of a substance like lecithin, the latter thus apparently acting like complement. Furthermore, it has been shown that the lecithin unites directly with the toxin, forming the so-called cobra lecithid. In this case, accordingly, the venom molecules act like amboceptors. These facts are highly suggestive in themselves, but it would be unjustifiable without direct evidence to generalize from them with regard to serum complements, which are of a very different nature from substances of definitely known chemical constitution like lecithin. The fact that an anti-immune-body, which has a comparatively specific action on the immune-bodies of the species of animal whose serum has been used in its development, produces its effect by preventing the union of complement, would appear to indicate that the anti-immune-body acted by filling up the complementophile groups of the immune-bodies (amboceptors). There are, however, difficulties in accepting this comparatively simple

[1] Kyes, *Berlin. Klin. Woch.*, 1902, nos. 38, 39 ; 1903, nos. 42, 43.

explanation, to which further reference is made below (p. 113).

In favour of the view that complement ultimately unites with the cell receptors is the fact that immune-body can be dissociated after saturation with complement. The progressive character of the lysis which may occur when there is a sub-hæmolytic dose of separable immune-body as contrasted with what is seen in the case of a sub-hæmolytic dose of fixed complement, points in the same direction. The matter is fully discussed below (p. 37), but we may here state that in view of all the results obtained, we believe that immune-body which has led to the union of complement can be in part recovered, while the complement cannot, owing to its having become firmly united to the cell receptors. Although we have stated that direct union of immune-body and complement cannot be regarded as having been demonstrated, we have, on the other hand, direct evidence that a certain amount of complement combines directly with receptors, e.g. those of tissue cells, bacteria, and even with heated red blood-corpuscles (p. 24). According to the view that the immune-body acts as a sensitizer, this might be due to its producing in other receptors affinity for complement, or to its increasing the affinity of the molecules already possessing it in some degree. The former would appear to be the more probable, in view of the firmness of the direct union of complement with tissue cells. It is further to be noted, that although differences in the combining groups of complements from the same animal can be made out, there is certainly nothing like the specific relationships which obtain in the case of the cytophile group of immune-bodies. The supposed specific properties of complements of different animals as judged by their anti-complements has now been shown to depend in great part at least on the phenomena of deviation of complement described below (p. 133). We have also found that complements from different animals

exclude one another in hæmolytic combinations, and have thus apparently a similar haptophore group; in fact, as we have elsewhere stated, there is a certain community or general character in the combining relationship of different complements. We may further point out that some of the differences observed in complements are of quantitative rather than of qualitative nature. For example, various bacteria absorb bactericidal complement as tested on a particular organism before they take up hæmolytic complement, but if a sufficient amount of bacterial emulsion be used the hæmolytic complement will also be absorbed. This matter of the degree in the combining affinities has been to a large extent overlooked by workers on the subject. The sensitizing of a molecule, therefore, does not imply the creation of a new combining group corresponding to the supposed special characters of the haptophore group of complement, but merely the production of some change in a molecule which allows a substance (complement) with very general combining affinities to enter. It is also shown below that the nature of the receptor is an important factor in determining whether or not complement will be taken up after the union of immune-body. In the case of sensitized ox's corpuscles, the maximum absorption of ox's complement is almost reached with one dose of immune-body; subsequent addition of *similar molecules* of immune-body, though these enter into combination with the receptors, does not lead to increased absorption of complement. This result must depend upon the receptors. Although the question as to the constitution and mode of action of immune-body cannot be considered to be completely settled, we believe, in view of all the facts that complement is brought into union with the cell receptors as a result of the action of immune-body. The existence of a special complementophile group in the latter is not proved, and the use of the term ' amboceptor ' does not appear to be justified.

SECTION B. ON THE PROPERTIES OF COMPLEMENT AND ITS MODIFICATIONS

On the Direct Union of Complement with Cells

In some preliminary observations on the behaviour of complement, I found that it united directly with various animal and vegetable cells, a fact which I afterwards found had been recorded by von Dungern[1] a considerable time previously. Of such cells may be mentioned those of the various organs of the same animal as that supplying the complement or of animals of different species, various bacteria, yeasts, &c. Von Dungern supposes that this combination takes place through the complementophile group of the amboceptors when they are still in the position of side-chains in the cells. Whether this is the explanation or not need not be discussed at present; the important point is that molecules with direct affinity for the same complement are very widespread in the animal and vegetable kingdom. It is generally stated that red corpuscles, unlike other cells, have no affinity for complement, and it is the case that no appreciable amount of complement is taken up by the amount of red corpuscles commonly used in hæmolytic experiments. If, however, the stromata be obtained from a large amount of blood and tested, it is easy to demonstrate that a considerable amount of complement enters into combination. I have obtained the stromata by shaking the blood with water, then adding sufficient chloride of sodium to make up to 0·8 per cent., and then centrifugalizing and separating the sediment. This process is repeated several times and the stromata are obtained as an almost colourless viscous mass. I have used the stromata both after they have been freshly prepared and after they

[1] Von Dungern, *Münchener Medicinische Wochenschrift*, 1900, No. 20.

have been kept for some time in the dry condition. Furthermore, if washed red corpuscles be heated for twenty-four hours at 55° C. and then their absorptive properties for complement be tested, it is found that quite an appreciable amount of the latter enters into combination. The following facts may be stated regarding this direct union of complement.

1. The combination is a firm one and I have been unable to recover the complement after it has been taken up. The test is made by washing in normal salt solution the cells, &c., which have taken up complement, to rid them of free complement, and then bringing them into intimate contact with red corpuscles treated with immune-body, the mixture being exposed for the usual time at 37° C. No hæmolysis occurs.

2. When the cells, &c., which have taken up complement are exposed to a temperature sufficient to destroy free complement—i. e. 55° C.—or rather sufficient to deprive complement of its characteristic property, the affinity for complement is not restored. This may be interpreted in accordance with Ehrlich's views on the constitution of complement, according to which it possesses a labile zymotoxic group and a more stable haptophorous group. The action of heat destroys the zymophorous group though apparently not the haptophorous (*vide* section on complementoids). The result which has been stated as to the effect of heating on the combined complement shows that the latter, when presumably it is altered by heat, is not displaced by normal complement.

3. The direct union of complement with cells, &c., takes place most rapidly about 37° C., whereas at 0° C. practically no combination occurs. This can be readily tested by the usual methods. In this respect the direct union of complement corresponds with the union through the intervention of immune-body.

On the Nature of the Union of Complement through the Medium of an Immune-body

Having thus found that complement enters into direct combination of firm nature with various cells, bacteria, &c., we have to consider the nature of the combination of complement brought about through the medium of an immune-body. In this connexion there are two chief subjects to be considered, namely, (*a*) the degree of firmness of the union, and (*b*) the amount of complement which is absorbed in the case of varying amounts of immune-body. A simple experiment demonstrates at once the firmness of the union, and the increased amount of complement fixed, by increasing the amount of immune-body. We take two tubes, A and B, each containing 1 c.c. of suspension of red corpuscles; to A we add one dose of immune-body, and to B seven doses of immune-body; to each tube four doses of complement are added. The tubes are placed in an incubator for two hours at 37° C.; lysis is of course complete in both tubes at the end of that time. 1 c.c. of suspension of sensitized corpuscles is added, and the tubes are once more placed in the incubator. The result is that the added corpuscles in tube A become completely lysed, whilst those in tube B are unaffected. This shows that the larger amount of immune-body in tube B has led to the combining of all the complement which was added (four doses). It also shows that the combined complement is not separated by dissociation in the conditions of the experiment, otherwise some lysis of the added corpuscles would have occurred. Numerous experiments of similar character have been made, and though it cannot be stated absolutely that no dissociation of complement ever occurs, one can say that even when large amounts of complement have been taken up, the separation of an

appreciable amount cannot be satisfactorily demonstrated. In one experiment twenty doses of complement were added to 1 c.c. of a 20 per cent. suspension of red corpuscles along with sufficient immune-body, and after suitable time for combination 1 c.c. of sensitized corpuscles was added. There was no lysis of the latter to be noticed, although in the conditions of experiment the separation of one-fifth of a dose of complement would have been detected. Accordingly, in this example we can say that there was no dissociation of even 1 per cent of the complement which had combined. Another opportunity of judging with regard to this question is given when there has been a small quantity of complement added in excess, so that a fraction of the sensitized corpuscles which are added undergoes lysis. We can place the tubes aside for twenty-four hours at room temperature, and observe whether there is any marked increase of lysis, such as might occur through the dissociation of the combined complement. In the case of the immune-serum, *rabbit v. ox*, along with guinea-pig's complement, I have not found any distinct evidence of dissociation of complement by this method. In other cases, for example in the case of the immune-serum, *rabbit v. guinea-pig* along with rabbit's complement, we do find that the lysis increases under the conditions mentioned. This might be due to dissociation of complement, but it might also be due to the presence of very weakly acting complements which are present in the serum, the effects of which are only seen after some time.

It is sufficient to state that in most cases of hæmolysis the combination of complement is a firm one, though the possibility of dissociation of small quantities in certain cases is not to be denied. A similar statement applies to the union of complement and bacteria by means of the corresponding immune-body. In the case of the absorption of a complement by a serum and its anti-serum (p. 143) we sometimes meet with phenomena which suggest dis-

sociation of complement; that is, a late lysis occurring in the test corpuscles after time has been allowed for the combination of complement with the serum + anti-serum. This is the case with the absorption of guinea-pig's complement by guinea-pig's serum with anti-serum from the rabbit, and is referred to in our original paper as a ' weak combination of complement with anti-complement '. Here again the possibility of there being weakly acting complement molecules, which are not taken up by the serum + anti-serum, cannot be entirely excluded, but the probability appears to be that there is some dissociation of complement. Unequivocal proof of the dissociation of complement could only be supplied if we could recover complement from cells, bacteria, precipitates, &c., with which it had combined, after these were repeatedly washed and centrifugalized, and this we have not yet succeeded in doing. The firmness of union of complement or the fixation of complement in most cases is of the greatest importance in serum researches, and really constitutes the basis of a number of the methods employed. No absolute statement can be made, however, and the behaviour of complement must be studied in connexion with any particular combination under consideration.

The Amount of Complement absorbed through the Medium of Immune-body

We have now to consider the exact amount of complement which is taken up in proportion to the amount of immune-body combined with the red corpuscles. For any given amount of immune-body a series of test-tubes, conveniently about nine, is taken; each tube contains 1 c.c. of suspension of red corpuscles along with the stated amount of immune-body; to the several tubes we add gradually increasing amounts of complement. (We can judge by experience what is the likely amount of complement to be taken up, and arrange the amounts so that in the first

tube there is considerably less than this, and in the last tube considerably more.) The tubes are then placed in the incubator for two hours at 37° C. At the end of this time the combination of complement is practically complete. To each tube is then added a cubic centimetre of sensitized red corpuscles, and the tubes are once more placed in the incubator for an hour and a half and allowed to stand in a cool place till next morning. The results are then read. By this method we can observe (*a*) the tube in which the added corpuscles have undergone the smallest perceptible degree of lysis, and (*b*) the tube in which they are completely lysed. The lysis occurring in the added corpuscles might theoretically be due either to free complement or to complement which had become dissociated. As above stated, however, there is no evidence that with the combination used in the experiments the latter occurs to any appreciable degree, and accordingly any lysis may be accepted as being due to complement which has been left free. In testing whether or not any lysis has occurred in the added corpuscles it will be found a convenient method to pipette off the supernatant fluid, the non-lysed corpuscles having formed a sediment at the bottom of the tube ; to each tube can then be added 1 c.c. of distilled water, and the amount of corpuscles can then be tested by the colorimetric method. The fraction of surviving corpuscles subtracted from unity will of course give the amount of lysis which has occurred.

Of a large number of experiments of this kind performed the following may be cited as examples. The immune-body used in these was obtained from the rabbit by injecting with ox's corpuscles (*immune-body, rabbit v. ox*). The complement was normal guinea-pig's serum, and was always treated with ox's corpuscles at 0° C. to remove the normal immune-body. For convenience, only six tubes which give the required results are quoted, though as a rule more were used.

EXAMPLE 1, SERIES (*a*).—1 c.c. 5 per cent. suspension of corpuscles and 1·5 doses of immune-body in each tube.

Amount of complement.	0·08	0·09	0·1	0·11	0·12	0·13 c.c.
Lysis in corpuscles added after two hours' incubation	none	slight	distinct	marked	almost complete	complete

SERIES (*b*).—1 c.c. suspension of corpuscles and 3 doses of immune-body in each tube.

Amount of complement	0·16	0·18	0·2	0·22	0·24	0·26 c.c.
Lysis in corpuscles added after two hours' incubation	none	distinct	marked	complete	complete	complete

M.H.D. of complement = 0.0475 c.c.

If we judge of the amount of complement taken up by the point at which free complement first appears, we find that 1·5 doses of immune-body take up, approximately, 0·085 c.c. of complement: 3 doses of immune-body take up, approximately, 0·17 c.c. of complement.

EXAMPLE 2. Details as before.

SERIES (*a*).—1 c.c. of 5 per cent. suspension of corpuscles and 1 dose of immune-body in each tube.

Amount of complement	0·025	0·03	0·035	0·045	0·05	0·06 c.c.
Lysis in added corpuscles	none	trace	marked	complete	complete	complete

SERIES (*b*).—1 c.c. of 5 per cent. suspension of corpuscles and 5 doses of immune-body in each tube.

Amount of complement	0·1	0·12	0·14	0·16	0·18	0·2 c.c.
Lysis in added corpuscles	none	slight	complete	complete	complete	complete

M.H.D. of complement = 0·017 c.c.

Result.—1 dose of immune-body takes up 0·027 c.c. of complement. 5 doses of immune-body take up 0·11 c.c. of complement.

EXAMPLE 3. Details as before.

SERIES (*a*).—1 c.c. of 5 per cent. suspension of corpuscles and 1 dose of immune-body.

Amount of complement	0·03	0·04	0·05	0·06	0·07	0·08 c.c.
Lysis in added corpuscles	none	trace	distinct	marked	complete	complete

SERIES (*b*).—1 c.c. of suspension of corpuscles and 7 doses of immune-body.

Amount of complement	0·175	0·2	0·225	0·25	0·275	0·3 c.c.
Lysis in added corpuscles	none	none	slight trace	almost complete	complete	complete

M.H.D. of complement = 0·017 c.c.

Result.—1 dose of immune-body takes up 0·035 c.c. of complement. 7 doses of immune-body take up 0·21 c.c. of complement.

EXAMPLE 4. Details as before. Here only the results need be given.

2 doses of I B took up 0·04 c.c. of guinea-pig's complement.
11 „ „ 0·195 c.c. „ „
13 „ „ 0·22 c.c. „ „

M.H.D. of complement = 0·015 c.c.

Numerous other experiments have given corresponding results, and as much as twenty or even more doses of complement may be taken up when high multiples of immune-body are used.

The general result is that up to a certain point each dose of immune-body leads to the taking up of additional complement, and if a few, say three or four, doses of immune-body are present the amount of complement combined is almost proportionate. With a greater number of doses, however, the amount is proportionately not quite so great. We have to bear in mind, however, that all the immune-body added does not enter into combination, a fraction remaining free in the fluid, and this naturally will not lead to the union of complement. With eight doses of immune-body, for example, nearly one dose remains free (*vide supra*);

thus the amount of combined complement will be diminished. Even if this allowance be made, there is a certain falling-off in the additional amount of complement taken up for each additional dose of immune-body when higher multiples are used. In the case of rabbit's complement the amount taken up is proportionately less than in the case of guinea-pig's complement, especially with the higher multiples of immune-body.

The facts stated refer to the serum *rabbit v. ox.* Results with othe rsera are given incidentally below (pp. 71–83). The following may be quoted in illustration :—

Experiment XLII. *Immune-body, Rabbit v. Guinea-pig.*

2 D (doses) of IB took up 0·08 c.c. guinea-pig's C.
8 ,, ,, 0·288 ,, ,,

Experiment LX. *Immune-body, Rabbit v. Guinea-pig.*

1 D of IB took up 0·2 c.c. rabbit's C.
3 ,, ,, 0·45 ,, ,,
10 ,, ,, 1·16 ,, ,,

Experiment LXII. *Immune-body, Guinea-pig v. Rabbit.*

1 D of IB took up 0·04 c.c. of guinea-pig's C.
10 ,, ,, 0·36 ,, ,,

Experiment LXIII. *Immune-body, Guinea-pig v. Rabbit.*

1 D of IB took up 0·22 c.c. of rabbit's C.
10 ,, ,, 1·12 ,, ,,

Experiment LXXIII. *Immune-body, Rabbit v. Ox.*

1 D of IB took up 0·04 c.c. of ox's C.
4 ,, ,, 0·05 ,, ,,
8 ,, ,, 0·05 ,, ,,

This last combination forms an exception to the general rule, as here almost the maximum amount of combined complement is obtained with a little more than one dose of immune-body (p. 83).

As a rule, therefore, multiple hæmolytic doses of immune-body combined with red corpuscles lead to the union of increased amounts of complement, and in some instances the comple-

ment taken up is approximately proportional to the amount of immune-body; in other cases considerable divergence is met with. It is thus seen that the dosage of complement necessary for lysis, just as in the case of immune-body, gives no indication of the amount which can be taken up; complete lysis may represent quite an early stage of the total combination possible.

The Ehrlich Phenomenon in the Union of Complement

In the earlier experiments with small multiples of the hæmolytic dose of immune-body it almost appeared that when complement was added there was an exact neutralization point in which the further addition of a dose of complement gave one dose free, but on using higher multiples, and especially with certain samples of serum, it was found that after the appearance of free complement much more than a lytic dose had to be added before complete lysis of the added corpuscles was obtained. It may be stated as the result of a large series of experiments that the Ehrlich phenomenon is always present, though sometimes, especially in the case of very active sera, it may be very slight. Examples of the manner in which the phenomenon appears are seen in the tables above. It may be stated that the same features in the mode of combination of complement are seen in the case both of serum receptors+their anti-substances and of bacteria+the homologous immune-bodies. And in the latter case it is noteworthy that we have been unable to find any dissociation of complement after it has combined.

In the case of the union of immune-body with receptors of red corpuscles, the Ehrlich phenomenon has been explained above as being due to the reversibility of the combination, but in the case of the union of complement this cannot be the explanation, as the reversibility is either non-existent or

it is of trifling degree. As a result of numerous experiments, we have come to the conclusion that it is due to variations in the combining affinities of the complement molecules of the serum, as Ehrlich has maintained with regard to the composition of diphtheria toxin. Some of the complement molecules are weaker, both as regards their combining affinities and their hæmolytic action, than others ; that is, they correspond to the epitoxoids. This weaker moiety of complement will accordingly be left over in any combining experiment and will thus account for the prolongation of the interval between the first appearance of free complement and the presence of a free hæmolytic dose. This supposition will also explain the great variations of the degree to which the phenomenon is observed in the case of different sera; whereas any explanation seems to be impossible on the supposition that all the complement molecules are the same. Furthermore, the extreme example of a complementoid is got when the serum is heated at 55° C. so as to abolish entirely its hæmolytic action, as will be shown below (p. 47). One can still demonstrate in such a heated serum the presence of molecules which retain in part their characteristic combining properties and prevent the union of complement. The addition of serum heated at 55° C. to fresh serum should increase or lengthen out the phase of incomplete lysis in the added corpuscles, that is, should make the Ehrlich phenomenon more marked, and this is found to be the case. While giving the above as the explanation, we fully recognize that there may be other factors concerned, as the combination of organic molecules like those in question may involve principles which are not yet understood.

On the Separation of Immune-body after Saturation with Complement

If immune-body forms, as Ehrlich believes, a link between the receptor of the red corpuscle and complement, then seeing that complement is not separable after combination, saturation with complement should lead to the locking-up of the molecules of immune-body so that they can no longer be separated. Various experiments have been carried out in order to test whether this is the case, and these show that immune-body is still recoverable after the treatment with complement. To take an example :— nine doses of immune-body are added to 1 c.c. of suspension of red corpuscles, and after time is allowed for combination they are centrifugalized; the fluid is pipetted off, and they are washed several times in salt solution. To the sensitized corpuscles there is added a considerable excess of complement; that is, more than will be taken up through the medium of the immune-body; the tube is then put in the incubator for two hours at 37°, and then to the clear fluid we add 1 c.c. of untreated corpuscles. It is found that complete lysis of these takes place. In spite, therefore, of the saturation with complement, sufficient immune-body for lysis has been obtained by dissociation. We can modify this experiment as follows :—After the sensitized corpuscles have been treated for two hours with excess of complement, we can remove this excess by adding a quantity of bacterial emulsion (say, of v. Metchnikovi) treated with its corresponding immune-body. This will absorb the complement left free, and accordingly when fresh corpuscles are then added no lysis occurs, but if thereafter they be separated by centrifugalization and washed in salt solution, and then complement is added, lysis occurs. This result shows, as before, that they have obtained immune-body by dissociation. (In these experiments the guinea-pig's serum

to be used must be completely freed from the natural immune-body for ox's corpuscles, which it possesses in varying amounts. This is of course done by adding washed ox's corpuscles to the serum for an hour at 0° C., and then centrifugalizing.)

Having thus seen that it is not possible to completely lock up the molecules of immune-body combined with the receptors of the corpuscles by means of excess of complement, we have now to inquire whether the complement really diminishes the amount of immune-body which can be separated. In this case we compare the amount of immune-body which is recoverable, in one instance before the addition of complement, in the other after the saturation with complement. We proceed as follows :—Two series (A and B) of nine tubes, each containing 1 c.c. of the standard suspension of red corpuscles, are taken and increasing amounts of immune-body are added to these, say from two doses in the first tube up to ten doses in the ninth tube ; time is allowed for combination of the immune-body, the tubes are centrifugalized and the corpuscles are washed several times in salt solution. All the immune-body is thus in combination. We then proceed further :—

Series A. To each tube is added 1 c.c. of suspension of red blood corpuscles ; the tubes are shaken, and are placed in the incubator for an hour at 37° C. ; to each tube four doses of complement are added, and the tubes are replaced in the incubator for an hour. The result is read on the following morning. (Complete lysis of all the first set of corpuscles of course occurs, and lysis of the second in proportion to the amount of immune-body which has been dissociated.)

Series B. To each tube is added more complement than can be taken up through the medium of immune-body (this amount can be calculated by means of other experiments) ; the tubes are then placed in the incubator for

two hours at 37° C., so as to allow fixation of complement; to each tube is added 1 c.c. of untreated corpuscles, and the tubes are replaced in the incubator for another hour. (As there is free complement present, lysis of the added corpuscles will again be proportionate to the amount of immune-body got by dissociation.)

The result of such an experiment is that the amount of lysis in the two series is practically the same; in the tubes containing originally from ten down to six doses complete lysis of the added corpuscles has taken place; whilst in the other tubes partial lysis has occurred; in the five-dose tube almost complete lysis, in the four-dose tube distinct lysis, in the three-dose tube very slight lysis, and in the two-dose tube no appreciable lysis. The procedure has also been varied by estimating the amount of immune-body which may be obtained (*a*) in one case after saturation with complement, (*b*) in the other case after lysis of the corpuscles with a single dose of complement. Here again the result is practically similar. The amount of lysis in the two series closely corresponds. In further experiments with other sera, the results were not always identical, in some cases there being rather more dissociation of immune-body before the addition of complement than after the saturation. The differences were, however, in no case great.

These results have an important bearing on the question as to the amboceptor constitution of immune-body. Manifestly, if immune-body forms a link between the molecules of complement and the receptors of the red corpuscles, then theoretically it should not be possible to dissociate immune-body without complement. As we have seen, however, under the conditions of the experiments, complement is not dissociated to any appreciable extent, nor is the combination *immune-body + complement* separable; on the other hand, immune-body is always obtained after the addition of complement in excess. There are, however,

two points to be borne in mind: the one is (which we had not recognized till after the experiments were first performed) that immune-body at 37° C. separates to a certain extent into the surrounding fluid. It is thus possible that a certain amount of immune-body may have become free before the combination of complement was complete. Even, however, on this supposition, we would expect the amount of immune-body dissociated to be much diminished by the fixation of complement. Then, again, we do not know that every molecule of immune-body takes up a molecule of complement, and theoretically it is possible that immune-body attached to a receptor A of a corpuscle may not take up complement, and thus be free to dissociate; whereas it may be active in hæmolysis (that is, take up complement) when attached to receptor B in the same or another corpuscle. Study of this kind shows clearly that we can regard neither the receptors, nor the molecules of immune-body, nor the molecules of complement as being identical in their respective classes—it is not as if we were dealing with the combination of three definite chemical substances. We do not maintain that the results completely disprove the theory that the immune-body acts as an amboceptor; but the experiments on the whole are against such a view, and at least point strongly to the fact that ultimately the molecule of complement becomes firmly attached to the receptor of the corpuscle. It is to be noted that even, however, if immune-body was no longer dissociable after the union of complement, this in itself would not establish its amboceptor constitution; it would simply prove that its union had become firmer after the union of complement.

Another point bearing on this subject may be referred to. If it is the case that immune-body can be dissociated from the receptors of the red corpuscles after having led to the union of complement, whereas the complement is firmly fixed, then the following phenomenon should result.

When a sub-hæmolytic dose of immune-body is present along with excess of complement, lysis should progress to some extent after the usual time necessary for the union of complement ; that is, some of the immune-body molecules which have led to the union of complement should become dissociated, and attach themselves to other receptors, increased union of complement and increased lysis thus resulting. On the other hand, with a sub-hæmolytic dose of complement lysis should have practically reached its maximum after the time stated, all the complement present having become fixed. As a matter of fact, this is what happens in the case studied, namely, immune-body *rabbit v. ox* along with guinea-pig's complement. We take a series of tubes, each containing the standard amount of corpuscles, and add to the several tubes increasing fractions of immune-body up to a little more than the hæmolytic dose, along with excess of complement in each tube ; if we then observe the amount of lysis after one and a half hours' incubation at 37° C., and again after the tubes have been kept for eighteen hours longer in a cool chamber at about 12° C., we find that in the latter case lysis has progressed considerably, complete lysis occurring with about two-thirds of the amount of immune-body which produced complete lysis after incubation. On the other hand, if we use complement in the same way with excess of immune-body, we find that there is scarcely any difference between the lysis in the two cases ; there is some increase after eighteen hours, but it is of quite a trifling nature. This result also appears to distinctly support the view that molecules of immune-body may dissociate after they have led to the union of complement. There seems to be only one other explanation of the results obtained, namely, that complement by itself can act on partially damaged corpuscles, but we have no evidence in support of such a supposition.

ON THE COMBINING PROPERTIES OF COMPLEMENTS

The combining properties of serum-complement have been studied in two chief relationships. In the first place, its combination with red corpuscles or bacteria, through the medium of the appropriate immune-body, has been investigated to a certain extent, and facts of considerable importance have been obtained. Taking the case of hæmolytic sera, we may for the present put aside the question whether the immune-body acts as a link (amboceptor) between the molecule of the red corpuscle and complement (Ehrlich's view), or whether complement enters into direct combination with the molecules of the red corpuscles through the influence of the immune-body. If we represent the combining molecules or receptors of the red corpuscles by R, and the immune-body by IB, it is sufficient for our present purpose to recognize that C (complement) combines with R + IB. In the second place, the action of a supposed anti-complement on complement has been studied. It was found that when the serum of an animal of species A was injected into one of species B, the serum of the latter acquired the property of inhibiting the action of the complement of the former, and Erhlich and Morgenroth[1] showed that this occurred by the haptophore group of complement becoming filled up, so that the complement could no longer combine with the sensitized corpuscles and produce lysis. According to this view, then, there was developed an anti-C, which neutralized C. As the anti-complement action appeared even when the serum injected had been heated at 55° C., Ehrlich supposed that only the zymotoxic group of complement was destroyed by heat, the haptophore group remaining ; in other words, that complement is converted into complementoid on heating, and that the latter also has the property of giving rise

[1] Ehrlich and Morgenroth, *Berlin. Klin. Woch.*, 1900, No. 31.

to anti-complement. The action of various anti-complements obtained in this way was found to be comparatively specific—they had always the greatest effect on the complement of the animal whose serum had been injected, though sometimes also a slight action could be demonstrated on the sera of allied species. The work of Moreschi and others, however, subsequently showed that the anti-complement action referred to was capable of explanation in another way. The whole question is fully discussed below (p. 133), but we give here the fundamental facts. The serum injected in addition to containing complement also contains certain molecules or receptors which act as antigens and give rise to anti-substances. These serum receptors, when combined with their anti-substances, absorb or fix complement just as the receptors of red corpuscles or bacteria in combination with their immune-bodies do. If we indicate the serum receptors by S, then the absorption of complement is indicated by S + anti-S + C. Accordingly, when the serum is mixed with the anti-serum, complement becomes fixed according to the scheme given and thus an apparent anti-complement action results. All the facts known with regard to anti-complements seem capable of explanation in this way, and the existence of true anti-complements, that is, of simple anti-substances which combine directly with complement, has not yet been established. The important work of Bordet [1] on anti-complements, with his application of the results to the toxin-antitoxin question, and various other researches, must be considered according to the new interpretation.

In our own investigations on the haptophore or combining group of complement we had before us the following facts difficult of explanation. Guinea-pig's and rabbit's complement combine equally well with sensitized ox corpuscles, and produce lysis—therefore their haptophore

[1] Bordet, *Annal. de l'Inst. Pasteur*, vol. xvii, 1903, p. 161.

groups appear to be the same. On the other hand, 'anti-complement' for guinea-pig's complement has very little effect on rabbit's complement—therefore the haptophore groups of the two complements appear to be different. We found on investigating the matter that, in the case studied, the combination of complement with sensitized corpuscles was much firmer than its combination with 'anti-complement'. In fact, with the latter there was evidence of some dissociation of complement after combination. We considered that this difference in the firmness of union might explain the difference in the two cases, and said, 'It is quite intelligible that differences shown to exist where the chemical union is of a loose nature may not be detectable when the combining affinity is strong.' The facts, however, can be readily explained according to the new views regarding anti-complement action. If the anti-serum obtained by injecting guinea-pig's serum is added to fresh guinea-pig's serum, we have all the conditions for deviation of complement, seeing that both the serum receptors and the corresponding anti-substances are present. If, however, we add the anti-serum to rabbit's complement, it is manifest that the specific serum receptors are practically absent; therefore the complement is not absorbed—the anti-complement action is apparently absent. We can, however, add the serum receptors as a minute quantity of heated guinea-pig's serum, and then we find that rabbit's complement is fixed, just as guinea-pig's is; in other words, the anti-complement becomes effective on other complements when a small quantity of the homologous serum is added to the mixture. This is readily intelligible according to the scheme S + anti-S + C. We have omitted a number of our experiments on this point, as they have not now the significance which we supposed them to have. We may, however, refer to one point which we investigated, namely, as to whether the guinea-pig's complement combines with the same molecules in the sensitized

red corpuscles as rabbit's complement does; in other words, as to whether these two complements from different species of animals possess similar haptophore groups.

As is well known, ox's corpuscles, treated with immune-body from the rabbit, are lysed on the addition either of guinea-pig's complement or of rabbit's complement. This would point to the haptophore groups of the two complements being the same. Ehrlich and Morgenroth have supposed in the corresponding case of the immune-body to rabbit's corpuscles obtained from the guinea-pig, that there are really two immune-bodies present, one of which combines with the rabbit's complement and one with the guinea-pig's, and point out in favour of this view that when rabbit's complement is used, the minimum hæmolytic dose of immune-body is about ten times greater than when guinea-pig's complement is used. We have accordingly investigated the case of the immune-body obtained by injecting the rabbit with ox's corpuscles. As both rabbit's and guinea-pig's complements are taken up by ox's corpuscles combined with immune-body, the question comes to be whether both complements combine with the same molecules. Does the combination of guinea-pig's complement prevent the combination of rabbit's complement and vice versa? We have conducted a large number of experiments of this nature, and the result has always been to give an answer in the affirmative. The experiments are on the same lines as those already described. Suppose we wish to test how much guinea-pig's complement is kept from combination by the previous combination of a given amount of rabbit's complement. To one series (A) of tubes, each containing the test amount of corpuscles combined with the same amount of IB (generally 3–4 doses), we add increasing amounts of guinea-pig's complement and test how much the treated corpuscles will take up. In another series (B) of similar tubes, we add a given amount of rabbit's C, allow

combination to take place for an hour at 37° C., and then test as before how much guinea-pig's C will be taken up. (It is convenient in such experiments to produce lysis first in all the tubes, in a case such as the present by guinea-pig's C. The amount of C used for initial lysis, of course, is added to the final result.) The excess in the amount of guinea-pig's C taken up in series A over that taken up in series B gives the amount which has been kept out by the amount of rabbit's C used. The following may be cited as examples :—

1. No. 3. M.H.D. of guinea-pig's C = 0·05 c.c.; M.H.D. of rabbit's C = 0·22 c.c. About 3 D of IB added to each of two series of tubes, each tube containing 1 c.c. suspension of red corpuscles.

Series A. Guinea-pig's C alone added. Amount taken up = 0·24 c.c.

Series B. 0·25 c.c. rabbit's C first, and then guinea-pig's C. Amount of guinea-pig's C taken up = 0·19 c.c.

Therefore, 0·25 c.c. of rabbit's C has kept from combination about 0·05 c.c. guinea-pig's C.

2. No. 24. M.H.D. of guinea-pig's C = 0·04 c.c.; M.H.D. of rabbit's C = 0·3 c.c. About 3 D of IB added to each tube as before.

Series A. Rabbit's C alone added; amount taken up = 1·35 c.c.

Series B. 0·04 c.c. of guinea-pig's C first, and then rabbit's C. Amount of rabbit's C taken up = 0·9 c.c.

Therefore, 0·04 c.c. of guinea-pig's C has kept out 0·45 c.c. rabbit's C.

3. No. 31. M.H.D. of guinea-pig's C = 0·0175 c.c.; M.H.D. of rabbit's C = 0·1 c.c., 4 D of IB added to each tube.

Series A. Guinea-pig's C alone added. Amount taken up = 0·11 c.c.

Series B. 0·1 c.c. of rabbit's C first, and then guinea-pig's C. Amount of guinea-pig's C taken up = 0·09 c.c.

Therefore, 0·1 c.c. rabbit's C has kept out 0·02 c.c. guinea-pig's C.

In all our experiments, of which the three cited are only examples, the result has been the same—guinea-pig's complement keeps out rabbit's complement and conversely rabbit's complement keeps out guinea-pig's.

Another method is to determine whether R + IB molecules saturated, say, for rabbit's complement are also saturated for guinea-pig's complement. To one series (A) of tubes, each containing the same amount of red corpuscles and the same amount of IB, complement of rabbit is added in increasing amounts, and the tubes are placed in the incubator for two hours at 37° C. The same procedure is carried out in another series (B), but at the end of the two hours a hæmolytic dose of guinea-pig's C is added to each tube. The tubes of both series are placed in the incubator for another hour, and the presence of free C is tested for in the usual way. It is evident that the first series will give the point of saturation with rabbit's C, and the corresponding tube in the second series will show whether such a tube can still take up guinea-pig's complement. As an example, in one experiment it is found that in series A the tube with 0·2 c.c. rabbit's C added gives a fifth of a hæmolytic dose of free C, whilst in series B the tube containing 0·2 c.c. rabbit's C gives a full hæmolytic dose of free C. Thus it is shown that the tube saturated with rabbit's C took up not more than a fifth of a dose of guinea-pig's C. (A separate estimation carried out at the same time gave 0·14 c.c. as the amount of guinea-pig's C taken up by the same amount of red corpuscles treated with IB.) It will be shown below that a corresponding result is obtained by saturation with complementoids.

We have, therefore, shown that in the case studied practically all the molecules of the guinea-pig's complement combine with the same R + IB molecules (sensitized receptors) of the ox as the molecules of the rabbit's complement combine with.

Another interesting point which presents itself is whether the hæmolytic value of a complement corresponds with the combining value. The hæmolytic dose of normal rabbit's serum in the case studied is always several times that of guinea-pig's complement. This theoretically may be because there are fewer complement molecules in a given amount of

rabbit's serum or because the zymotoxic group of the rabbit's complement is less active than that of the guinea-pig's complement. In the former case a hæmolytic dose of rabbit's complement will prevent the combination of a hæmolytic dose of guinea-pig's complement; in the latter it will prevent the combination of more. Our experiments are not sufficiently extensive to give a definite statement on this point, especially in view of the fact that during the progress of an experiment the hæmolytic action of complement may diminish, and this change in the value does not always occur in the two complements in the same proportion. We may say, however, that we have obtained in several instances a correspondence between the combining and the hæmolytic ratio, i. e. a hæmolytic dose of guinea-pig's complement keeps out a dose of rabbit's, and vice versa. We are inclined to think that this may be the rule in the case before us, and that probably the divergences in these ratios which we have also met with are the result of accidental disturbing causes. We do not consider this point as satisfactorily settled. It is to be noted that the M.H.D. of the IB is practically the same with the two complements used, provided that the natural IB of the guinea-pig's serum for the ox's corpuscles be first removed. The marked difference in the dosage of the two complements would therefore depend upon there being more complement molecules in the guinea-pig's serum active towards the ox's corpuscles than in the rabbit's serum, rather than upon differences in toxicity of the two complements. In other cases described below this does not hold; on the contrary, a great difference in the relative toxicity of the complements of different animals is brought out (p. 85).

On Complementoids and their Combination

Ehrlich has pointed out the analogy which exists between complements and toxins of various kinds. Looked at from the point of view of the red corpuscle or bacterium, the complement is the toxic agent which leads to its partial or complete destruction, the auxiliary action of the immune-body being, however, necessary. From his study of changes which occur in toxins and their neutralization by anti-toxin, he came to the conclusion that the toxophore group might undergo degeneration while the haptophore group survived, though its combining energy might be weakened. To such an altered toxin he gave the name toxoid. The results of hæmolytic studies led him to the conclusion that a similar change might occur in complements, the result being complementoids. The chief evidence for the existence of complementoids is the following :—

In most cases when normal sera are heated to 55° C. for an hour, the characteristic action (hæmolysis, bacteriolysis) of complement is lost, but such a serum when injected into an animal of different species has the faculty of leading to the formation of anti-complement. Hence, apparently the zymotoxic group of the complement has been destroyed, while the haptophore remains. (As mentioned above (p. 40) this argument has now lost its validity, since it has been shown that receptors in the serum injected give rise to anti-substances and the combination of these two absorbs or fixes complement (*vide* also p. 133).) Again, Ehrlich and Sachs [1] showed in the case of the hæmolytic action which the dog's serum normally possesses towards the guinea-pig's corpuscles, that the combining groups of sensitized corpuscles might be filled up by complementoid on treating with heated serum, and thus the action of complement, i. e. hæmolysis, might be prevented. In most instances, however, this is

[1] Ehrlich and Sachs, *Berlin. Klin. Woch.*, 1902, No. 21.

not the case, and we must conclude either that complementoid does not usually combine with R + IB, or that if it does, it can be displaced by complement. So far as we know, the existence of complementoids in such heated sera has not been shown by test-tube experiments. We accordingly bring forward the following methods which prove their existence :—

Is Complementoid Present in Serum Heated at 55° C. ?

We have demonstrated the existence of complementoids by two methods, viz. : (1) by showing that they combine with R + IB molecules after lysis, and thus prevent a certain amount of C from being taken up ; (2) by showing that they combine with a serum + its anti-serum (with S + anti-S), and thus diminish the amount of C which can be taken up by such a combination. And, further, the variations in the dosage of complement necessary to produce lysis when the corpuscles are suspended in various heated sera instead of salt solution seem only explicable on the supposition of the existence of complementoids. We may state that the complement to be tested has usually been heated for $1\frac{1}{4}$ hours at 57° C., and in every case a test was made, to show that it was devoid of hæmolytic power ; we shall represent such heated serum by Cd (complementoid).

1. As was shown by Ehrlich and Morgenroth, the addition of Cd to red corpuscles treated with IB does not prevent the combination of C and the occurrence of lysis. We may represent this as follows, the small circle indicating a red corpuscle :—

$$\bigcirc \left\{ \begin{matrix} \text{R} \\ \text{IB} \end{matrix} + \text{Cd} \right. \quad \vdots \quad + \text{C} \quad \vdots \quad \text{Result} = \text{lysis.}$$

The vertical dotted line indicates *a period of incubation for one hour*, unless when otherwise stated.

After lysis has occurred, however, Cd can combine with

the R + IB molecules. It has been shown that a large amount of C can be taken up through the medium of multiple doses of IB, and that the combination of C is a firm one. Thus :—

	2 hrs.		
$O4\left\{\begin{matrix}R\\IB\end{matrix}\right.+4C$ Lysis	+	$O\left\{\begin{matrix}R\\IB\end{matrix}\right.$	Result = no lysis in added corpuscles, because all the C has been taken up.

If, however, we add in the same case 3Cd after lysis by 1C, but before the addition of the other 3C, the result is different, thus :—

		2 hrs.		
$O4\left\{\begin{matrix}R\\IB\end{matrix}\right.+C$ Lysis	+ 3Cd	+ 3C	$+O\left\{\begin{matrix}R\\IB\end{matrix}\right.$	Result = lysis in added corpuscles, because some of the C has been kept out by Cd.

In other words, the Cd molecules have united with the R + IB molecules after lysis, and have prevented the added C from being taken up ; this is shown by the test corpuscles undergoing lysis by means of the free C.

To avoid repetition, we may here state that the stages of all such experiments are the following :—

1. To each of a series of tubes containing the standard amount of corpuscles, a certain amount of IB is added, usually three to four hæmolytic doses.

2. To each tube is added a little more than the hæmolytic dose of complement, and a certain amount of complementoid (heated serum). The tubes are placed in the incubator for an hour at 37° C. Hæmolysis, of course, occurs, and time is allowed for the Cd to combine with the R + IB molecules.

3. To the several tubes in series complement is then added in increasing amount. Experience shows how much C is likely to be taken up, and the last tubes should, of course, contain more than this. The tubes are incubated at 37° C. for two hours.

4. To each tube is added the standard amount of red corpuscles, treated previously with 3 D of IB, and the tubes are placed in the incubator for another hour. Hæmolysis will, of course, take place in the added corpuscles according to the amount of uncombined C present in each tube. The tubes are then placed in a cool chamber till next morning; the red fluid in each tube is pipetted off, and the amount of sedimented corpuscles left is estimated as above described.

In a control set of tubes complement only is added, and we thus ascertain how much C is taken up when no Cd is present. It is then seen how much C has been prevented from combining with the R + IB molecules by means of a given amount of Cd.

2. Complementoid combines with a serum + its anti-serum,[1] and prevents the union of complement.

If the ordinary action of such a combination is shown thus,

$$\text{S} + \text{anti-S} + \text{C} \quad + \text{O} \left\{ \begin{matrix} \text{R} \\ \text{IB} \end{matrix} \right. \quad \text{Result} = \text{no lysis.}$$

then we may represent the effect of Cd by the following scheme—

$$\text{S} + \text{anti-S} + \text{Cd} \quad + \text{C} \quad + \text{O} \left\{ \begin{matrix} \text{R} \\ \text{IB} \end{matrix} \right. \quad \text{Result} = \text{partial lysis.}$$

The following are the details of such an experiment :—

Two series (A and B), of nine tubes, each containing 0·5 c.c. salt solution.

C of guinea-pig, M.H.D. = 0·03 c.c., Cd = the same serum heated. Anti-S is the serum (heated at 57° C.) of a rabbit injected with guinea-pig's serum.

Series A. Each tube receives 0·1 c.c. anti-S and 0·1 c.c. Cd.

[1] This was stated in the original paper as 'complementoid combines with anti-complement, and prevents the union of the latter with complement' (*vide* p. 40).

Series B. To each tube, 0·1 c.c. anti-S alone is added.

All the tubes are placed in the incubator for an hour at 37° C.

To the several tubes of both series alike are then added increasing amounts of C, viz. 0·03, 0·04, 0·05 c.c., &c. Incubation for another hour. To each tube the standard amount of red corpuscles treated with IB is then added, and the tubes are incubated for another hour.

The results are shown in the following table :—

	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.
Amount of C added in c.c.	0·03	0·04	0·05	0·06	0·08	0·1	0·12	0·14	0·16	0·18
Series A— Amount of lysis in added corpuscles .	0·78	0·88	0·9	Almost complete			Complete			
Series B, ditto	0·1	0·55	0·62	0·7	0·77	0·80	0·88	Almost complete		Complete

The effect of Cd in preventing the combination of C is thus seen throughout. If we take the first tube, it is seen that the previous addition of 0·1 c.c. Cd has kept out sufficient C to produce lysis of 0·68 of the added corpuscles, i. e. about 0·02 c.c. C.[1]

[1] It is shown below that excess of the homologous serum interferes with the deviation of complement, and it might be suggested that the result detailed is not due to complementoid, but to the serum receptors added in the 0·1 c.c. heated serum. It is, however, to be noted that those serum receptors also exist in the unheated serum, and that there are as many in tube 9 of series B (0·16 c.c. of C) as in tube 4 of series A (0·06 c.c. of C + 0·1 c.c. of Cd). The deviation effect should thus be the same in both. But the actual amount of lysis is the same in both, though 9B contains 0·1 c.c. more C than 4A. This appears only explicable on the assumption that the Cd in 4A has prevented the C from combining.

On the Amount of Complementoid derived from Complement

We have seen that the evidence for the existence of complementoid is supplied by its preventing complement from combining with certain molecules (R + IB and S + anti-S respectively). The amount of complementoid present may be measured by the amount of complement which is thus kept out of combination. Accordingly, if each molecule of complement gives rise to one molecule of complementoid, then 0·1 c.c. of heated serum (Cd) should prevent the combination of the complement in 0·1 c.c. of the same serum unheated, provided, however, that the complement cannot displace the complementoid after it has combined. Suppose we wish to estimate how much complement will be kept out by a given amount of complementoid, we proceed as follows. Two series of tubes (A and B) containing 1 c.c. of a suspension of red corpuscles are taken, and to each tube is added the same amount of IB (say four doses) ; lysis is produced in all the tubes by a dose of C. To each tube in series A a given amount of Cd is added, and one hour at 37° C. is allowed for combination. We then add increasing amounts of C to the tubes in each series, and find, by the method described above, how much C is taken up in the two series. The difference between the amounts in the two series gives, of course, the amount of C which has been prevented from combining by the Cd used. We can in the same way compare the amount of guinea-pig's C kept out by a given amount of rabbit's C and Cd respectively, and the amount of rabbit's C kept out by guinea-pig's C and Cd. Of course, in every experiment of this kind, the Cd is a heated portion of the same C as that used for comparison.

A considerable number of experiments of this kind have been performed both with rabbit's and guinea-pig's com-

plementoids, and differences are found in the two cases. In five experiments in which rabbit's complementoid was used, it was found that there was kept out of combination a quantity of complement approximately equal to the amount of complementoid used. In the case of the guinea-pig's complement, on the other hand, the amount of complement kept out was always distinctly less ; on the average, 0·6 c.c. of C corresponded to 1 c.c. of complementoid. We may therefore say that a molecule of rabbit's C gives rise to a molecule of Cd, which has an affinity for the R + IB molecules after lysis, practically equal to that of C; whereas, with the guinea-pig's serum, in the process of heating either some of the C becomes entirely destroyed, or the Cd formed has a lower combining affinity and some of it can be displaced by C.

On the Relative Firmness of Union of Complement and Complementoid respectively with R + IB Molecules (Sensitized Receptors) after Lysis

To determine this, we have compared in the usual way the amount of unaltered C which can be taken up with the amount of a mixture of C and Cd in equal parts. If the C and Cd molecules have the same combining power, it is evident that the saturation of the R + IB molecules will occur after the addition of the same amounts in the two cases. After the saturation point has been reached, however, twice as much of the C + Cd mixture as of the undiluted C would have to be added before a free dose of C would be obtained. As a matter of fact, this is pretty much what happens.

The following may be taken as examples :—

I. Two series of tubes, A and B, three doses (3 D) of IB added to each tube, and lysis is produced in all by 1 D of guinea-pig's C, M.H.D. = 0·05 c.c. C is then added in increasing amounts.

A

	1.	2.	3.	4.	5.	6.	7.
Amount of C added in c.c.	0·1	0·15	0·2	0·25	0·3	0·35	0·4
Amount of C left over in doses	0	0	trace	much	1D+	1D+	1D+

B.

Amount of C and Cd mixed in equal parts	0·1	0·15	0·2	0·25	0·3	0·35	0·4
Amount of C left over in doses	0	0	first trace	trace	more	1D−	1D−

II. 5 D of IB to each tube. M.H.D. of C = 0·03 c.c.

A.

	1.	2.	3.	4.	5.	6.	7.	8.	9.
Amount of C added in c.c.	0.08	0·12	0·16	0·2	0·24	0·28	0·32	0·36	0·4
Amount of C left over in doses	0	0	0·65D	1D+	1D+	1D+	1D+	1D+	1D+

B.

Amount of mixture of C and Cd, equal parts added	0·08	0·12	0·16	0·2	0·24	0·28	0·32	0·36	—
Amount of C left over in doses	0	0	0·17D	0.65D	1D+	1D+	1D+	1D+	—

It appears from these examples that the points at which free C appears in the two cases ((*a*), where pure C is used, and (*b*) where a mixture of C and Cd in equal parts is used) approximately correspond—only a little more of the mixture of C and Cd has to be added before saturation of the R + IB molecules occurs. It is also seen that, after the saturation point has been reached, fully twice as much of the mixture as of pure C has to be added before a free dose of C is obtained. Of course, if the C molecules had a much greater affinity for sensitized receptors than the Cd molecules, the *actual* amount of C added would be approximately the same in the two instances. This, however, is very

far from being the case. In other experiments we have found, when lysis is produced first, then a small quantity of Cd added and allowed to combine, and then increasing amounts of C, that the surplus C appears to come off less sharply than when C alone has been added. This would appear to indicate that a certain amount of Cd is displaced by C ; it must, however, be a small amount.

We may therefore conclude that the C and Cd have *approximately the same* combining affinity for the R + IB molecules after lysis. If there is a difference in favour of the energy of combination of the former, it is a slight one.

On the Relative Firmness of Union of Complement and Complementoid with a Serum + its Anti-serum

Here the mode of procedure is of the same nature. A given amount of anti-S is added to salt solution in two series of tubes. To the A tubes increasing amounts of pure C are added ; to the B tubes the same amounts of a mixture of C and Cd in equal parts. One and a-half hours at 37° C. are allowed for combination, and then red corpuscles treated with IB are added to each tube to find how much C is obtainable. Examples :—

1.—0·1 c.c. anti-S in each tube.

A.

	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.
Amount of C added in c.c.	0·04	0·05	0·06	0·07	0.08	0·09	0·1	0·12	0·14	0·16	0·18
Amount of C obtainable from the mixture in doses (D)	0	0	0	first trace	0·12	0·27	0·27	0·4	0·73	0·82	0·88

B.

Amount of C and Cd in equal parts added	0·04	0·05	0·06	0·07	0·08	0·09	0·1	0·12	0·14	0·16	0·18
Amount of C obtainable in doses (D)	0	0	0	0	0	first trace	0·15	x[1]	0·3	0·55	0·68

[1] This tube was accidentally broken.

2.—0·05 c.c. anti-S in each tube.

A.

	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.
Amount of C added in c.c. ..	0·03	0·04	0·05	0·06	0·07	0·08	0·09	0·1	0·12	0·14
Amount of C obtainable in doses (D)	0·09	0·12	0·4	0·48	0·65	0·73	0·9	1·0	1+	1+

B.

Amount of C and Cd in equal parts added ..	0·05	0·06	0·07	0·09	0·1	0·12	0·14	0·16	0·18	0·2
Amount of C obtainable in doses	0·05	0·15	0·28	0·4	0·5	0·73	0·88	1	1+	1+

In this case of course, as already explained, the surplus C comes off much more gradually, and a more accurate result might be expected. The theoretical consideration of all the factors, however, is one of much complexity, and we do not claim to explain it fully. The following points are to be noted, however :—(1) The amount of C obtainable has been estimated by the amount of lysis—the two, however, only approximately correspond—it is not possible to detect the smallest amount of C, we can only note the first appreciable lysis ; (2) as has been stated, the combining value of C (fresh serum) usually falls somewhat when converted into Cd (unfortunately a separate estimation of the exact value of Cd in the experiments before us was not made) ; (3) when there is a surplus of C and Cd molecules the former are taken up by the intact corpuscles treated with IB, the latter very slightly ; the balance of dissociation may thus be affected.

Looking, however, at the results in a general way, we see that C is kept out of combination by Cd, that the points at which surplus C is obtained are not far distant from each other in the two series, and that the interval from the point at which C is first obtainable to that at which a full dose is

got is much lengthened in series B, though it is not quite doubled. If we take the tube in series B (mixture of C and Cd added), which gives a third lysis in the added corpuscles, we find that the corresponding tube in series A (C alone added) gives about two-thirds lysis. We may thus conclude that in this case also the firmness of union of Cd does not differ greatly from that of C.

On the Union of Complementoid with R + IB Molecules before Lysis

As has been mentioned above, the addition of complementoid to red corpuscles treated with IB does not prevent the subsequent action of complement and the production of lysis. This may be due either (*a*) to complementoid failing to combine with the R + IB molecules, or (*b*) to its being displaced by complement after it has combined ; both factors may be partly concerned. There are two methods by which the question may be investigated, and we have carried out both of them.

In the first place, we may add a given amount of heated serum (Cd) to red corpuscles treated with IB, allow time for combination, then remove the fluid by centrifugalization, and then test the amount of Cd present in the fluid by finding how much C it will prevent from combining with R + IB molecules after lysis. We shall call the Cd treated in this way Cdx. A control is made with the same amount of heated serum (Cd) which has not been in contact with red corpuscles treated with IB. We have performed a number of experiments of this kind, and the result has always been to show that only a fraction of the Cd combines with the R + IB molecules. In one experiment it was found that the Cdx of the rabbit kept out two-thirds of the amount of guinea-pig's C kept out by rabbit's Cd, i.e. only one-third of the Cd molecules had combined with the intact red

corpuscles treated with IB. With guinea-pig's Cdx, in one case a similar result was obtained, whilst in another a half of the Cd molecules had been removed by combination.

Another method is to bring Cd into contact for a given time with red corpuscles treated with IB (say, 3 IB), then to centrifugalize and wash these corpuscles, and then test how much C they will take up. The control will be red corpuscles treated with the same amount of IB, but not with Cd. Similar results emerge from experiments of this kind. The following may be taken as an example :—

Three sets of tubes containing the standard amount of red corpuscles, fully 3D of IB added to each tube.

1. Series A.—We estimate how much guinea-pig's C will be taken up.

2. Series B.—We estimate how much C will be taken up after the addition of 0·04 c.c. Cd (lysis having been first produced by a dose of complement).

3. Series C.—The corpuscles are treated with 0·6 c.c. Cd for an hour at 37° C., then centrifugalized and washed to remove the free Cd. We then estimate how much C the corpuscles will take up.

The result is that in—

Series A.....	0·175 c.c. of C	is taken up.
„ B.....	0·15 „	„ „
„ C.....	0·165 „	„ „

In other words, after lysis the addition of 0·04 c.c. of Cd has kept out 0·025 c.c. of C, whereas before lysis the addition of 0·6 c.c. of Cd (fifteen times the amount) has only kept out 0·015 c.c. of C. It is thus seen that a mere fraction of Cd entered into combination with the R + IB molecules before lysis. We may add that the largest amount observed was in an experiment with rabbit's Cd, where it came out that of 0·25 c.c. of Cd added before lysis an amount was taken up equal to 0·1 c.c. of Cd added after lysis.

From all these experiments it is shown that before lysis Cd has a weak affinity for R + IB molecules, and that in any given case only a fraction of the amount added enters into combination. This result is of high importance in connexion with the general question as to the action of these substances, since it implies that the action of one substance—complement—increases the combining affinity of another substance—complementoid.

On the Saturation of the R + IB Molecules with Complementoids, after Lysis has occurred

Seeing that red corpuscles treated with multiple hæmolytic doses of IB take up multiple doses of C, we may produce lysis by a single dose of C, and then find whether the affinities of the remaining R + IB molecules can be satisfied with Cd. The different stages of such experiments are : (*a*) The addition of several doses of IB to the corpuscles, time being allowed for combination ; (*b*) Lysis by a single dose of C ; (*c*) The addition of Cd in excess ; two hours at 37° C. are allowed for combination ; (*d*) The addition of about one dose of C ; one hour at 37° C. for combination ; (*e*) The addition of red corpuscles treated with IB to test for the presence of free C. (In case the hæmolytic value of C should fall during the experiment, the same amount of C as that used in (*d*) should be added to salt solution and placed in the incubator at the same time for an hour ; its value is then tested by adding red corpuscles treated with IB.) We can in this way test the saturating power of rabbit's or guinea-pig's Cd with rabbit's or guinea-pig's C—four possible combinations. A considerable number of experiments of this kind have been carried out, but the general result may be stated that after saturation with Cd not more than a fraction of a dose of the added C has been taken up.

Examples 1 and 2.—*Saturation with Rabbit's Cd. Test with Rabbit's and Guinea-pig's C.*

M.H.D. of IB = 0·002 c.c.
„ guinea-pig's C = 0·045 c.c.
„ rabbit's C = 0·11 c.c.

(0·5 c.c. of suspension of red corpuscles is used, so that the doses will be half the amount.)

5 D of IB added to four tubes, A, B, C, D.

Lysis in all with 0·07 c.c. rabbit's C.

0·6 c.c. rabbit's Cd added to each tube; two hours at 37° C., then add

A.	B.	C.	D.
0·065 c.c.	0·09 c.c.	0·03 c.c.	0·05 c.c.
Rabbit's C.		Guinea-pig's C.	

One hour in incubator.

Add to each 0·5 c.c. of suspension of red corpuscles treated with IB.

The result is that complete lysis of all the added corpuscles occurs. This shows that at most not more than a fifth of a M.H.D. of rabbit's C and not more than a third of a M.H.D. of guinea-pig's C has been taken up.

3. *Saturation with Guinea-pig's Cd. Test with Guinea-pig's C.*

M.H.D. of guinea-pig's C = 0·04 c.c. (at beginning of experiment), 5 D of IB added to 1 c.c. corpuscles, lysis with C and addition of 0·5 c.c. of Cd, two hours at 37° C.; 0·06 c.c. of C added, one hour at 37° C.; red corpuscles treated with IB added, one hour at 37° C. Result, 0·8 of the corpuscles underwent lysis, but in a control with 0·06 c.c. of C alone 0·1 remained undissolved. Here, accordingly, not more than about a tenth of a dose of C subsequently added entered into combination with the R + IB molecules.

4. *Saturation with Guinea-pig's Cd.*
Test with Rabbit's C.

M.H.D. of guinea-pig's C = 0·07 c.c.
,, rabbit's C = 0·27 c.c.

Three tubes taken containing 1 c.c. suspension of red corpuscles, treated with 6 IB.

Lysis with 0·09 c.c. guinea-pig's C ; one hour in incubator. Addition of 0·5 c.c., 0·75 c.c. and 1 c.c. of guinea-pig's Cd to the several tubes ; two hours in incubator.

Addition of 0·3 c.c. rabbit's C ; one hour for combination.

Addition of the test amount of corpuscles treated with IB. Result in the tube with 0·5 c.c. Cd, two-thirds of the corpuscles underwent lysis ; in the other tubes with 0·75 c.c. and 1 c.c. Cd, complete lysis took place. Complete saturation had not occurred with 0·5 c.c. Cd, but with the other amounts it had, so that *at most* about a tenth of a dose of the rabbit's C was taken up.

It is thus seen that it is possible to practically saturate with complementoid the surplus R + IB molecules after lysis. Other experiments have indicated that probably a certain amount of complementoid is displaced by complement added afterwards. But the above results show that when an *excess* of complementoid is used, the amount displaced is reduced to a trifling amount.

These results also confirm the result already obtained, namely, that in the case under consideration the haptophore groups of the rabbit's and guinea-pig's complements unite with the same R + IB molecules (*vide supra*).

Conclusions

The following are the chief results obtained from the experiments described. It is, of course, to be understood

that they are held to apply only to the cases investigated, viz. the immune-body for ox's corpuscles obtained from the rabbit, used along with rabbit's and guinea-pig's complements and complementoids. Further observations will be necessary to determine whether they obtain generally.

1. The existence of complementoids in heated sera can be shown in ordinary test-tube experiments, by their preventing (*a*) the union of complement with a serum + its anti-serum (formerly styled 'anti-complement'); (*b*) the union of complement with R + IB molecules after lysis.

2. The amount of complementoid derived from complement, as tested by the combining relationships, varies; in the case of the rabbit it is approximately equal to the original amount of complement; in the case of the guinea-pig it is considerably less than that amount.

3. The combining affinity of complementoid, both for a serum + anti-serum and for R + IB molecules after lysis, is not much inferior to that of complement.

4. On the other hand, complementoid has a feeble affinity for R + IB molecules before lysis, i.e. for intact red corpuscles treated with immune-body; of the complementoid added only a small quantity enters into combination; hence complementoid does not prevent lysis by complement.

5. When red corpuscles united with multiple doses of immune-body are lysed by a single dose of complement, the surplus R+IB molecules can be saturated with excess of complementoid, so that almost no complement can subsequently be taken up. This result is obtained also with rabbit's complementoid and guinea-pig's complement, and with guinea-pig's complementoid and rabbit's complement.

ON COMPLEMENTOIDS IN RELATION TO THE DOSAGE OF COMPLEMENT IN DIFFERENT MEDIA[1]

The basis of the following observations was a phenomenon recorded by Bordet[2] in connexion with anti-immune-bodies. He found that the anti-immune-body might be able to protect corpuscles treated with immune-body against the action of complement when the corpuscles were suspended in guinea-pig's serum heated at 55° C., (i.e. deprived of complement) whilst the protective action might fail when they were suspended in salt solution. In other words, the action of the complement on the corpuscles was greater in the latter medium. We have made corresponding observations and can fully confirm Bordet's results. In explanation of the phenomenon he considers that the salt solution is a less suitable medium for the corpuscles than the heated serum of the guinea-pig, thus the hæmoglobin diffuses out more readily. There is thus only a relative neutralization of the immune-body—' la sensibilisation simplement atténuée produit encore ses effets si les globules sont maintenus dans un milieu diminuant leur résistance.' He further considers that the fact referred to is somewhat analogous to the observations of Roux and Vaillard that a mixture of tetanus toxin and anti-toxin might be harmless for normal guinea-pigs and still be dangerous for guinea-pigs previously debilitated by vaccination against the cholera vibrio. We have considered the subject of some importance and have carried out observations with the following results. It may be pointed out, however, that the further application of Bordet's explanation would imply that ox's serum heated at 55° C. is a less suitable medium for ox's corpuscles

[1] This section was originally part of the paper " On the Properties of Anti-Immune-Bodies and on Complementoids," by R. Muir and C. H. Browning, *Journ. of Hygiene*, vol. vi, 1906.

[2] Bordet, *Annales de l'Inst. Pasteur*, vol. xviii, p. 593.

than guinea-pig's serum heated at 55° C., as the hæmolytic dose of complement is less in the former medium.

It is to be noted at the outset that the corpuscles used are those of the ox, the immune-body is obtained from the rabbit by injecting it with ox's corpuscles, and the complement is that of the guinea-pig. It seemed to us a somewhat curious circumstance that the heated serum of the guinea-pig should be a specially suitable medium for suspending the corpuscles of the ox, and have considered it desirable to test the hæmolytic dose of complement when the corpuscles are suspended in different media, especially in the heated sera of different animals. Such heated sera, though bereft of complement or rather of the toxic action of complement, contain complementoids according to Ehrlich's view; this matter will be referred to below. So far as we know no observations of this kind have been made.

It is scarcely necessary to state that all the experiments were carried out with the different media of suspension at the same time and for the same periods of time. The results may be given in tabular form, and the doses are most conveniently given in terms of the minimum hæmolytic dose in salt solution.

Ox's corpuscles. Immune-body from Rabbit. Guinea-pig's complement.

Medium of suspension.	*Hæmolytic doses of complement necessary for complete lysis.*
0·85 per cent. sodium chloride solution	1
Ox's serum 55°	2
Guinea-pig's serum 55°	5·5
Guinea-pig's serum 55° and ·85 per cent. salt solution, equal parts	3+
Rabbit's serum 55°	2+

N.B. The relative dosage varies considerably with different samples of serum, but the figures given may be taken as a fair average.

It appears clearly from this table that the dose of complement necessary for hæmolysis varies greatly according to the medium in which the corpuscles are suspended, and

we cannot offer in every case an explanation; no doubt factors of different kinds are concerned. We will confine our attention to the case of hæmolysis of the ox's corpuscles suspended in the heated serum of the guinea-pig. It will be seen that the dose of complement is in this case about three times the dose when the corpuscles of the ox are suspended in their own serum. In other words, the guinea-pig's serum would seem to protect the ox's corpuscles against the toxic action (complement) better than the ox's serum, or to be a more suitable medium in Bordet's sense. On theoretical grounds it appears that some other explanation must be looked for. It will also be seen that the dose of complement necessary even when the corpuscles are suspended in a mixture of equal parts of salt solution and guinea-pig's serum 55° is greater than when the corpuscles are in their natural medium.

We have inquired into the method by which the guinea-pig's serum 55° retards lysis, that is, demands a higher dose of complement. Theoretically there are at least two possibilities, *the one being that the heated serum in question interferes with the combination of complement, the other that it in some way retards the diffusion of hæmoglobin.* We have found that the former is the chief factor in bringing about the result. If the corpuscles be suspended in heated serum of the guinea-pig and 3 M.H.D. of complement be added (M.H.D. being the minimum dose in salt solution) only slight lysis occurs in the course of two hours at 37° C. Now if at the end of this time the surviving corpuscles be removed from the serum, then washed and suspended in salt solution and placed in the incubator for another period, the corpuscles do not undergo lysis. Consequently active complement has not entered into combination with them. (It is to be noted that of course multiple doses of immune-body were present to begin with.)

We have also shown that in such a case as that men-

tioned there is still *free complement* in the medium of suspension. We take two series (A and B) of tubes each containing 0·5 c.c. of suspension of red corpuscles along with five doses of IB ; the medium in A being salt solution, in B guinea-pig's serum heated at 55° C. To the several tubes in the two series increasing doses of complement are added. After lysis is complete in the tubes of series A we test as to the comparative amount of free complement in the two series. The fluid from each tube of series A is added to another tube containing 0·5 c.c. of guinea-pig's serum 55°, the fluid obtained by centrifugalization from each tube of series B is added to a tube containing 0·5 c.c. of salt solution (0·85 per cent.). In this way any free complement obtained from the tubes of either series is now in a mixture of equal parts of heated serum and salt solution. To each tube we now add the corpuscles of 0·5 c.c. of suspension treated with immune-body and the tubes are placed in the incubator. It is found that lysis takes place much more readily in the tubes containing the fluid from series B, i. e. there was much more free complement in this series. Such an experiment is complementary to and confirmatory of the result recorded above.

Having thus seen that the combination of complement with corpuscles treated with immune-body is *interfered with* when guinea-pig's serum 55° is the medium of suspension, we have to inquire how this is brought about.

Now it is to be noted that the guinea-pig's complement (normal serum) is much the most active of the complements used, and that this serum when heated interferes with hæmolysis more than the others. In a preceding section[1] we have given reasons for believing that there are more complement molecules in the guinea-pig's serum than (for example) in the same quantity of rabbit's serum. We have also given methods for demonstrating by test-tube

[1] *Vide* p. 45.

experiments the existence of complementoids in heated sera, these of course being derived from complements. Let us suppose that the hæmolytic dose of guinea-pig's complement for the test 1 c.c. of suspension of corpuscles in salt solution is 0·01 c.c. and that each molecule of complement gives rise to a molecule of complementoid. Then when the same amount of corpuscles is suspended in heated serum instead of salt solution and 0·01 c.c. of fresh serum is added, there will be a hundred molecules of complementoid for each complement-molecule—under this condition complete lysis does not occur. This is probably an exaggerated statement of the case, at least we were unable to show by combination tests that there were as many complementoid molecules as there were originally complement molecules. For the sake of illustration let us put the proportion at fifty complementoid molecules and one complement molecule. We have further shown that when complementoid is brought into contact with red corpuscles treated with immune-body only a small proportion of complementoid combines with them, so that when the corpuscles are afterwards washed and complement is added lysis is not interfered with. But it seems possible that when a large number of molecules with feebler affinity (complementoid) are actually present in the mixture, the combination of those with stronger affinity (complement) may be interfered with. Furthermore, when lysis occurs the combining affinity for complementoid molecules is much increased; complete lysis may thus be considerably interfered with by their presence. It therefore appeared reasonable to inquire what the effect would be if the complementoid molecules were *removed* from the serum. If our supposition is correct, then lysis should occur much more readily, i.e. with a smaller dose of complement.

Now the complement action may be removed from a serum in two ways, viz. (*a*) by heating at 55° C., and (*b*) by bringing the serum into contact with substances for which the com-

plement has a combining affinity. In the former case the complement is converted into inactive complementoid; in the latter, if the substance with which it is combined can be separated by centrifugalization, the complement will be actually removed from the serum. In the former case, if our theoretical considerations are correct, the serum when used as a medium of suspension will interfere with lysis; in the latter this interfering action should be absent. Acting on these ideas we have investigated how hæmolysis will progress in a serum from which the complement has been *removed*. We can remove the complement from a fresh serum by bringing it into contact with some cells or bacteria treated with the corresponding immune-body and then after time has been allowed for combination remove the serum by centrifugalization. In this way a serum practically free from complement as tested by hæmolytic experiments is obtained. We have carried this out by various methods, the most satisfactory of which is the following. Washed corpuscles of the ox in 0·85 per cent. salt solution are placed in a sterilizer overnight at 55° C., they are then centrifugalized and the brownish fluid is removed. The hæmolytic receptors of such heated corpuscles are, however, not destroyed; they still have the power of combining with immune-body and thereafter of taking up complement. To a suspension of heated corpuscles a large amount of immune-body is added, and after time is allowed for combination of the latter the corpuscles are repeatedly washed in salt solution and centrifugalized; finally the fluid of the suspension is removed as completely as possible, so as to avoid dilution of the serum. To the corpuscles thus treated the fresh serum of the guinea-pig is added and the mixture is thoroughly shaken up and placed in the incubator at 37° C. for two hours; the tubes are then centrifugalized and the serum is pipetted off. If sufficient amount of corpuscles and immune-body are used

the serum is rendered practically devoid of hæmolytic action; sometimes there remains a trace of complement which becomes evident when relatively large quantities are used, as is the case when it is employed as the medium of suspension of corpuscles. To get it entirely free of hæmolytic action we heat it for an hour at 55° C.; the small amount of complementoid which may thus remain does not interfere with the test. We shall speak of the serum thus freed of complement as 'treated serum', while serum heated at 55° C. will be designated 'serum 55°'. In addition to the method described we have also used, to take up the complement from the serum, red corpuscles combined with immune-body and freed from fluid. In this case, of course, the corpuscles when added to the serum undergo lysis, but by adopting certain procedures the hæmoglobin-stained serum can still be used as a medium of suspension. In other experiments we employed an emulsion of kidney cells along with the corresponding immune-body, the cells being afterwards removed by centrifugalization. All the results obtained have been of the same nature; but as the first-mentioned method is the most satisfactory we need give details only with regard to it.

In estimating the hæmolytic dose of complement in the 'treated serum' we used in each tube the corpuscles of 0·5 c.c. suspension, and of course 0·5 c.c. of serum was added after the salt solution had been removed by centrifugalization.

Doses of three samples of Guinea-pig's Complement in different Media of Suspension for the Corpuscles of 0·5 *c.c. Suspension.*

0·85 *per cent. sod. chloride.*	*Serum* 55°.	*Treated Serum.*
0·005	0·03 (6 D)	0·01 (2 D)
0·0125	0·06 (5 D)	0·02 (1·6 D)
0·004	0·1 (25 D)[1]	0·015 (3·75D)

It thus appears that the dose of complement is very much smaller when the medium of suspension is treated guinea-

[1] This was by far the largest dose observed in any of our experiments and must be regarded as exceptional.

pig's serum than when it is guinea-pig's serum 55°. In other words, the serum 55° exerts a strong inhibitory influence on lysis, which is not the case when the complement is removed from the serum instead of being changed into complementoid. The dose in guinea-pig's 'treated serum' is in fact little, if at all, greater than that in ox's serum 55°. The high dose of complement necessary when serum 55° is used as the medium of suspension was supposed by us to be due to complementoid, and this view is fully confirmed by the results of using as the medium of suspension the serum deprived of its complementoid. We are thus justified in concluding that the presence of a large amount of complementoid interferes with the action of complement and thus raises the hæmolytic dose.

These results are of importance in connexion with the question as to the existence of complementoids and their combining properties, and constitute an amplification and confirmation of what has been stated in the preceding section. Gay [1] in a recent paper criticizes the well-known experiment of Ehrlich and Sachs [2] in which the complementoid of dog's serum prevents the action of guinea-pig's complement on guinea-pig's corpuscles sensitized with the natural immune-body in the dog's serum, and comes to the conclusion that the supposed complementoid is merely an attenuated complement—attenuated both as regards its combining affinity and its toxic action. We would point out, however, that in our former experiments, as in the present case, the serum heated at 55° C. is quite devoid of hæmolytic action; in fact, so far as can be seen from the hæmolytic action, the complement has entirely disappeared. Nevertheless, in such a serum, a substance (complementoid) is present which

[1] Gay, *Centralbl. f. Bakteriol. u. Parasitenk.*, 1. Abt., Originale, Bd. xxxix, S. 172.

[2] Ehrlich and Sachs, *Berlin. klin. Wochenschr.*, 1902, No. 21. In a recent paper, *Centralbl. f. Bakteriol.*, 1. Abt., Originale, Bd. xl, S. 125, Sachs has replied to Gay's objection and confirmed his previous results.

combines with the same molecules as complement, viz. with the complex *receptors + immune-body*. We claim, in fact, that the existence of complementoids has been demonstrated by test-tube experiments, and that Ehrlich's views regarding these bodies have been completely confirmed. It would, in fact, be impossible to explain the high dose of complement necessary when the corpuscles are suspended in guinea-pig's serum 55° on the theory that complementoids are merely attentuated complements, i.e. attenuated in combining affinity and toxic action in equal degree.

Summary of results :—

1. The dosage of complement varies greatly according to the medium in which the red corpuscles are suspended.

2. The most striking variation observed was the very high dose of guinea-pig's complement necessary to produce lysis when ox's corpuscles are suspended in guinea-pig's serum 55°, a dose which is about six times the dose necessary in salt solution and about three times the dose in ox's serum 55°.

3. The high dose of complement necessary is chiefly due to the complement being prevented from entering into combination with the corpuscles treated with immune-body.

4. When the complement in guinea-pig's serum is removed by combination instead of being converted into complementoid by heating at 55° C., the dosage of complement in such a treated serum is much diminished and becomes approximately the same as in ox's serum 55°.

5. We conclude that the presence of a large amount of guinea-pig's complementoid interferes with the combination of complement, and the dose of the latter necessary for lysis is thus increased.

THE COMBINING PROPERTIES OF COMPLEMENTS IN RELATION TO THEIR TOXIC ACTION

Ehrlich has pointed out the similarity in the constitution of complements and of various toxins, and our own observations, as above detailed, strongly support his views. We may, in the study of hæmolysis, consider the complement as a toxin, the red corpuscles treated with the appropriate immune-body as the object on which the toxin is to act, and the hæmolysis as the indication of the toxic action. Ehrlich regards the complement as consisting of two chief atom-groups, the haptophore or combining group and the zymotoxic ; but in speaking of the action of sera he does not always carry out this distinction completely. For example, the efficiency of different complements, as tested by their hæmolytic or bacteriolytic effects, is often taken as evidence of the degree of chemical affinity between the complements and the immune-body. But it is manifest that theoretically a complement may combine perfectly through the medium of the immune-body, and yet produce little hæmolysis, owing to the absence of sensitiveness to the zymotoxic group—combination or 'complementing' may occur and yet hæmolysis be deficient or absent. The question which we have investigated is accordingly this—Where different complements differ in their action as shown by the dosage, both of complement and of immune-body required, does this depend upon differences in their combining affinities or upon differences in their toxicity ?

In working out this problem we have made use of three sera, viz. (*a*) the serum of the rabbit injected with ox's corpuscles, therefore hæmolytic towards ox's corpuscles ; (*b*) the serum of the rabbit injected with guinea-pig's corpuscles ; (*c*) the serum of the guinea-pig injected with rabbit's corpuscles. In each case the hæmolytic serum is deprived of its complement by heating at 55° C. and,

therefore, contains only immune-body; it is accordingly inactive until complement (i. e. normal serum) is added.

In the first place, we may give in tabular form the average dosage of the several complements with the different immune sera: the test amount of corpuscles being 1 c.c. of a 5 per cent. suspension in 0·8 per cent. sodium chloride solution.

Immune-body and corpuscles tested.	*Rabbit's complement.*	*Guinea-pig's complement.*	*Ox's complement.*
IB rabbit *v.* ox / Ox's corpuscles	0·15 c.c.	0·025 c.c.	∞
IB rabbit *v.* guinea-pig / Guinea-pig's corpuscles	0·15 „	0·3 „	0·03 c.c.[1]
IB guinea-pig *v.* rabbit / Rabbit's corpuscles	0·5 „	0·07 „	0·04 „

This table shows that in the cases studied the highest dosage of the complement of an animal occurs when used against its own corpuscles.

Dosage of Immune-bodies with different Complements.

Complement.	*IB rabbit v. ox.*	*IB rabbit v. guinea-pig.*	*IB guinea-pig v. rabbit.*
Rabbit's	0·003 c.c.	0·003 c.c.	0·15 c.c.
Guinea-pig's .	0·033 „	0·03 „	0.015 „
Ox's	∞	?[2]	0·02 „

The most striking facts brought out in this table concern the relative doses of immune-bodies with rabbit's and guinea-pig's complements respectively. They are: (*a*) in the case of an immune-body acting on the corpuscles of another animal (viz. ox's corpuscles), its dose with rabbit's comple-

[1] The normal serum of the ox has a strong hæmolytic action both on rabbit's and on guinea-pig's corpuscles. This is due to the presence of a natural immune-body, and it is not possible to remove this in the usual way by placing the serum in contact with the corpuscles at 0° C. We have, however, made allowance for this circumstance, and the dosage of complement has been calculated accordingly, and may be taken as substantially correct.

[2] We have not succeeded in getting a satisfactory estimation of the dose of this immune-body with ox's complement, owing to failure to remove the natural immune-body for guinea-pig's corpuscles in the ox's serum.

ment is practically the same as that with guinea-pig's complement; (*b*) in the case of the immune-body acting on guinea-pig's corpuscles, its dose is ten times greater with guinea-pig's complement than with rabbit's complement; and, conversely, in the case of the immune-body acting on rabbit's corpuscles, its dose is ten times greater with rabbit's complement than with guinea-pig's complement. It is also to be noted that the immune-body to ox's corpuscles does not bring about complete hæmolysis at all when the ox's complement is used.

These tables supply the hæmolytic doses of the different immune-bodies and complements; they do not, however, give us the facts with regard to their several combinations. In illustration of this we may mention that Ehrlich and Morgenroth,[1] finding that the dose of the immune-body to rabbit's corpuscles obtained from the guinea-pig was ten times higher (as shown in the table) when rabbit's complement was used than when guinea-pig's complement was used, supposed that there were really two immune-bodies, one present in large amount taking up guinea-pig's complement, and another present in small quantity taking up rabbit's complement. This is manifestly a satisfactory theoretical explanation, but we have to inquire whether it is supported by facts; as will be shown below, this is not the case.

We shall accordingly consider the *amounts of complement taken up* through the medium of different doses of immune-body in the several cases. It will be convenient to take first the second and third sera, as above arranged. The method employed for estimating the amount of complement taken up depends upon the firmness of union of complement, and has been described above (p. 28). We take the amount of complement absorbed as indicated by the point at which free complement is first obtainable when the complement is added in increasing amounts.

[1] Ehrlich and Morgenroth, *Berlin. klin. Woch.*, 1900, No. 31.

I. Immune-body to Guinea-pig's Corpuscles (obtained by injecting the Rabbit with these corpuscles).

(a) *With Guinea-pig's Complement—*

We may first compare the results when guinea-pig's complement is used with those when rabbit's complement is used. In the former case the dose of complement is very high ; in fact, more than ten times the amount of guinea-pig's complement sufficient to hæmolyse ox's corpuscles is necessary to hæmolyse its own corpuscles. This might be due to the fact that only a fraction of the complement molecules suited the immune-body to guinea-pig's corpuscles, or it might be due merely to weakness of the toxic action of the complement. If the former were the case, the presence of the uncombined complement would be shown by adding the corpuscles of another animal treated with the corresponding immune-body. The matter is put to the test by adding varying amounts of guinea-pig's complement to guinea-pig's corpuscles with their corresponding immune-body, and then after allowing two hours at 37° C. for combination, to test for the presence of complement by means of ox's corpuscles treated with their immune-body. If we use 1 D of immune-body to indicate the amount necessary to produce lysis when rabbit's complement is used, then 10 D will be the M.H.D. when guinea-pig's complement is used. The following are the chief results which we have obtained. It is to be noted that, of course, if less than 10 D of IB is added complete lysis does not occur with guinea-pig's complement, and in such cases the tubes are centrifugalized and the clear fluid is added to the indicator, i.e. ox's corpuscles treated with their immune-body. This indicator is specially suitable on account of the high sensitiveness of the corpuscles to guinea-pig's complement.

Such an experiment may be graphically represented thus—

Guinea-pig's O + n IB + x guinea-pig's C : + Ox's O + IB

the small circle indicating red corpuscles, the vertical dotted line a period of incubation at 37° C., n indicating a definite multiple of IB, and x varying amounts of C.

IB Rabbit *v.* Guinea-pig. Combination of Guinea-pig's C.

Experiment XLII.—

2 doses of IB took up 0·08 c.c. guinea-pig's C.
8 ,, ,, 0·288 ,, ,,
Dose of guinea-pig's C = about 0·3 c.c.

Experiment XLIII.—

1 dose of IB took up 0·026 c.c. guinea-pig's C.
5 doses ,, ,, 0·114 ,, ,,
10 ,, ,, ,, 0·254 ,, ,,
Dose of C = 0·25 c.c.

Experiment XLIV.—

1 dose of IB took up 0·014 c.c. guinea-pig's C.
14 doses ,, ,, 0·38 ,, ,,
Dose of C = 0·35 c.c.

Experiment LXIX.—

1 dose of IB took up 0·04 c.c. guinea-pig's C.
5 doses ,, ,, 0·28 ,, ,,
10 ,, ,, ,, 0·48 ,, ,,
Dose of C = 0·4 c.c.

From these results it is manifest that the large amount of guinea-pig's complement necessary to produce lysis combines completely with the guinea-pig's corpuscles treated with the corresponding immune-body, there being, up to a certain point, no complement left over to act on the test corpuscles, and the large dose of immune-body necessary is simply due to this amount being required to bring the necessary complement into combination with the corpuscles. The guinea-pig's complement has, therefore, a *weak toxic action* on guinea-pig's corpuscles, about ten times weaker than it has, for example, on ox's corpuscles.

Note.—The amount of complement taken up is calculated from the point at which free complement is obtainable after time has been allowed for combination. It will be noticed that the amount of complement taken up is approximately, though not strictly, proportional to the amount of immune-body present. The divergence is more marked when higher multiples are used, as has been noted in the case of another combination (p. 32); here also what has been described as the 'Ehrlich phenomenon', is seen to a slight extent. As, however, there is very little evidence of dissociation of complement after it has combined, the phenomenon in this case is due to some other cause, probably to the presence of complementoid.

(b) *With Rabbit's Complement*—

It will be seen from the tables that (*a*) the minimum hæmolytic dose of immune-body in this case is small—about a tenth of that necessary when guinea-pig's complement is used, and (*b*) that the dose of complement also is comparatively small, in fact, practically the same as that necessary for the hæmolysis of ox's corpuscles. The difference in the doses of rabbit's and guinea-pig's complements for ox's and guinea-pig's corpuscles respectively is thus very noteworthy. In investigating the amount of rabbit's complement taken up by means of multiple doses of immune-body, interesting and at first very puzzling results emerged. An experiment of this kind may be graphically represented as before, thus :—

Guinea-pig's ○ + n IB + x rabbit's C : Guinea-pig's ○ + IB.

In performing experiments of this kind we found that five or even ten doses of immune-body apparently led to the taking up of scarcely more complement than one dose of immune-body did; and, further, that the amount of complement apparently taken up seemed to become less the longer the test corpuscles were left in the fluid. Thus, for example, at the end of two hours, 0·15 c.c. of complement might appear to be taken up, and next morning only 0·05 c.c. It appeared, therefore, (*a*) that multiple doses of immune-

body did not lead to the taking up of corresponding multiple doses of complement, and (*b*) that the complement taken up appeared to dissociate again in part, though this phenomenon might possibly be due to the presence of some complement molecules with very slow action. When, however, we used as the indicator *ox's corpuscles* treated with their corresponding immune-body, quite different results were obtained. The scheme is now :—

Guinea-pig's ○ + *n* IB + *x* rabbit's C : + Ox's ○ + IB.

The following results will serve as examples :—

Experiment LX.—

1	dose of IB	took up	0·2	c.c.	rabbit's C.
3	doses „	„	0·45	„	„
10	„ „	„	1·16	„	„

The M.H.D. of C was only 0·1 c.c. before the experiment; it is possible that it may have increased subsequently.

Experiment LXVIII.—

2	doses of IB	took up	0·28	c.c.	rabbit's C.
5	„ „	„	0·58	„	„
10	„ „	„	0·74	„	„

The M.H.D. of C was 0·06 c.c.

It is thus seen that when ox's corpuscles suitably treated are used as the indicator, the amount of complement taken up increases as the amount of immune-body is increased, though there is a greater deviation from strict arithmetical proportion than when guinea-pig's complement is used.

The difference in the results obtained with the two indicators (guinea-pig's and ox's corpuscles respectively), is manifestly due to the fact that there is in the rabbit's serum a complement which acts on guinea-pig's corpuscles, and not on ox's corpuscles, and that this complement either becomes dissociated from the guinea-pig's corpuscles or combines in very small amount. On the other hand,

the *chief* complement present acts on both varieties, and its union is a firm one; even with this combination, however, the amount taken up appeared to diminish somewhat over night.

The fact already stated that, when guinea-pig's corpuscles were used as the indicator, additional doses of immune-body did not appear to lead to the taking-up of additional amounts of complement, raised the question whether there might not be two immune-bodies present, one of which acted with rabbit's complement, and one with guinea-pig's complement. Evidence of this was sought for by leaving the immune-serum in contact with the corpuscles for a time, then separating by centrifugalization, and thereafter testing the dose of the uncombined immune-body with rabbit's and with guinea-pig's complement respectively. It is evident that if two immune-bodies were present, and were taken up by the corpuscles in different proportions, then the relative doses of the separated fluid would become changed. Such an investigation is theoretically of simple nature, but it is difficult to carry it out exactly, owing to the fact that it is not possible to remove completely the natural immune-body from the rabbit's serum, i.e. to make this serum entirely devoid of hæmolytic action. In several experiments, however, allowance being made for this circumstance, it appeared that the relative doses with the two complements did alter in the way that the dose with guinea-pig's complement became relatively still higher, i.e. after the contact with the corpuscles there seemed to be immune-body molecules left, which acted with rabbit's, but not with guinea-pig's complement. This may mean merely that the molecules of immune-body vary in their combining affinities, and that those with the weaker affinity act with the more powerful complement (rabbit's). The question is one of great complexity, and we have not attempted a full solution, as it did not appear necessary for the purposes of the present research. Everything goes to show, however, that the great majority of the immune-body molecules act both with rabbit's and with guinea-pig's complement; and we found that the presence of a small amount of guinea-pig's complement kept out of combination a certain amount of rabbit's complement, and vice versa.

(c) *With Ox's Complement—*

The ox's complement is not a very suitable one to employ in this combination, as the natural serum of the ox has a very powerful hæmolytic action in itself, and it is only possible to remove a small proportion of the natural immune-body by contact experiments. Nevertheless, we have found that the dose of ox's complement, along with the immune-body to guinea-pig's corpuscles, is a small one, and there is no doubt that guinea-pig's corpuscles are very sensitive to the zymotoxic action of ox's complement.

When we come to investigate the combining affinities, we find that multiple doses of immune-body have very little effect on the amount of ox's complement taken up, the amount taken up by means of from four to eight doses, for example, being very little more than that taken up by means of one dose. This may be due to a true want of combining affinity on the part of the complement, or it may be due to the combination being a very loose one. It may be noted, however, that there is practically, in the course of twelve hours, no evidence of dissociation of complement after it has really combined. The following will serve as an example :—

Experiment LXXIV.—

1 dose of IB	took up	0·04	c.c.	of ox's	C.
4 doses	,,	,,	0·042	,,	,,
8 ,,	,,	,,	,,	,,	,,

The indicator was guinea-pig's corpuscles treated with immune-body, for which the dose of ox's complement was 0·02 c.c.

II. Immune-body to Rabbit's Corpuscles (obtained by injecting the Guinea-pig).

(a) *With Rabbit's Complement—*

This case is in many ways analogous to that of the immune-body to guinea-pig's corpuscles along with guinea-pig's

complement. In both cases the hæmolytic dose of immune-body is ten times greater when the complement of the animal whose corpuscles are being tested is used than when the complement of the other animal is used. This will be seen from the table. In this case, also, the dose of rabbit's complement is high, just as in the previous case the dose of guinea-pig's complement was. How much rabbit's complement is taken up when multiple doses of immune-body are used? The scheme of experiment is :—

Rabbit's $\bigcirc + n$ IB $+ x$ rabbit's C : + Ox's $\bigcirc$ + IB.

The following are some of the results :—

Experiment LXII.—

1	D of IB	takes up	0·14	c.c.	rabbit's C.
10	,,	,,	1·16	,,	,,

Experiment LXIII.—

1	D of IB	takes up	0·22	c.c.	rabbit's C.
10	,,	,,	1·12	,,	,,

Experiment LXVI.—

1	D of IB	takes up	0·086	c.c.	rabbit's C.
5	,,	,,	0·55	,,	,,
10	,,	,,	0·9	,,	,,

Dose of rabbit's C with rabbit's corpuscles = about 0·6 c.c. '1 D' = M.H.D. of IB along with guinea-pig's C. '10 D' = M.H.D. with rabbit's C (*vide* tables, p. 72).

It will be seen that there is no lack of combining-power on the part of rabbit's complement, and that the amount increases with the amount of immune-body, though considerable deviations from exact arithmetical proportions are met with. Sometimes more, proportionately, is taken up by ten hæmolytic doses than by one hæmolytic dose, sometimes less; we have met with the former phenomenon in several other experiments than that quoted, and are not able to give at present an explanation of it. Another point is that the hæmolytic dose of immune-body (expressed

as '10 D. of IB.') leads to the taking up of more than a hæmolytic dose of rabbit's complement. This phenomenon is, probably, related to the fact brought out by Morgenroth and Sachs,[1] that the M.H.D. of complement sometimes varies greatly, according to the amount of immune-body used, and, conversely, the M.H.D. of immune-body may vary greatly, according to the amount of complement. The explanation of this is also outside the scope of the present paper.

(b) *With Guinea-Pig's Complement*—

The combination of guinea-pig's complement may be exemplified by the following :—

Experiment LXII.—

1 D of IB took up 0·04 c.c. guinea-pig's C.
10 ,, ,, 0·36 ,, ,,

Experiment LXIII.—

1 D of IB took up 0·012 c.c. guinea-pig's C.
10 ,, ,, 0·27 ,, ,,

The indicator was ox's corpuscles treated with immune-body. Here we have variations corresponding to those noted above.

We have also found that with this immune-body a small quantity of guinea-pig's complement keeps out of combination a certain amount of rabbit's complement, and that saturation with rabbit's complement implies practical saturation for guinea-pig's complement also. A similar statement applies to rabbit's complement keeping out guinea-pig's complement.

(c) *With Ox's Complement*—

Owing to the lack of anti-serum, we were unable to study the combining relationships of ox's complement through the medium of this immune-body. This defect, however, cannot modify the main conclusions arrived at.

[1] Morgenroth and Sachs, *Berlin. klin. Woch.*, 1902, No. 35.

III. Immune-body to Ox's Corpuscles (obtained by injecting the Rabbit).

It will be seen from the tables above that the dose of immune-body is practically the same with guinea-pig's as with rabbit's complement. The dose of the latter complement is the higher, and in a previous section (p. 45), it was shown that this was probably due to a smaller number of complement molecules in a given volume of serum, rather than to a weaker action of the zymotoxic group. The combining relationships of the two complements have also been fully discussed there, so that it is unnecessary to repeat the results obtained. It is sufficient to say that they behave as regards combination in hæmolysis pretty much as if they had the same haptophore groups. We shall refer merely to the action of *ox's complement* along with the above immune-body.

With this combination it is usually impossible[1] to produce more than a fraction of lysis in the corpuscles (usually not more than a tenth), no matter how large amounts of immune-body and complement are used; in only one case did we get a considerable amount of lysis, about three-fourths. We never obtained complete lysis, however. In other words, the ox's serum does not 'complement'. Is this due to want of combining power of the ox's complement, or to the deficiency of toxic action? This question can be answered by finding the amount of complement taken up when varying amounts of immune-body are used.

The scheme is :—

$$\text{Ox's } \bigcirc + n \text{ IB} + x \text{ ox's C} \vdots \text{ guinea-pig's } \bigcirc + \text{IB}.$$

[1] This applies only to cases where the immune-body and the complement are added at the same time to the corpuscles, as is the usual procedure. If, however, the immune-body be added to the corpuscles an hour before the complement, lysis of the corpuscles sometimes results (*vide*, p. 87).

As lysis does not occur in the first stage, the contents of each tube are centrifugalized, and the fluid is added to the guinea-pig's corpuscles. The following results were obtained :—

Experiment LXXIII.—

1 D of IB took up 0·04 c.c. ox's C.
4 „ „ 0·05 „ „
8 „ „ „ „ „

Dose of ox's complement for guinea-pig's corpuscles = 0·03 c.c.

Experiment LXXIV.—

1 D of IB took up 0·014 c.c. ox's C.
4 „ „ 0·02 „ „
8 „ „ „ „ „

Dose of ox's complement for guinea-pig's corpuscles = 0·02 c.c.

From these it is evident (1) that a considerable amount of ox's complement is taken up by one dose of immune-body (i.e. by 1 M.H.D. as tested with rabbit's or guinea-pig's complement), but this amount of complement, which may be more than sufficient to produce complete lysis of rabbit's or guinea-pig's corpuscles, produces almost no lysis of the ox's corpuscles; and (2) that the total amount of complement which can be taken up is almost reached with one dose of immune-body, additional doses of immune-body scarcely increasing the amount. There is, of course, in this case, no possibility of the phenomenon being due to dissociation of complement after combination, as the ox's corpuscles are removed by centrifugalization before the guinea-pig's corpuscles are added, and, therefore, any complement obtainable must have been free in the fluid. Accordingly, we have here, again, an example of the relative non-sensitiveness of an animal's corpuscles to the action of its own complement when it is brought into union with

them by an immune-body. But, in addition, there is, unlike the two previous cases, a deficiency also in the combining power of complement beyond a certain point, or, in other words, only a small proportion of the molecules of the red corpuscles combined with immune-body (R + IB molecules) take up ox's complement.

The fact that in the case just described, only some of the R + IB molecules take up complement is of considerable theoretical importance. It is to be noted that almost all the R + IB molecules capable of taking up complement are present after the addition of one dose of immune-body, and that the subsequent addition of the same immune-body molecules scarcely increases the amount of complement taken up, though these molecules combine with the receptors of the red corpuscles. It would therefore appear that the failure on the part of an R + IB molecule to combine with complement is due in some way to the receptors and not to the immune-body. According to Ehrlich's theory the amboceptor (immune-body) has practically no affinity for complement in the free state, but acquires that affinity when combined with the tissue or bacterial molecule. But the result above stated would, according to the amboceptor hypothesis, imply that only some molecules capable of combining with immune-body give the latter affinity for complement. According to the view that the immune-body renders the tissue molecule capable of taking up complement, the explanation would simply be that some of the molecules of the ox's corpuscles have no combining-group for the ox's complement, though they enter into combination with immune-body. It is not possible on theoretical grounds to establish either of these hypotheses by the exclusion of the other, but whichever may be ultimately established, the importance of the nature of the tissue molecule or receptor in determining whether complement will be taken up or not is brought out with sufficient clearness.

The chief results may be summarized as follows :—

1. In the action of a complement there are two distinct factors, viz. (*a*) power of chemical combination, and (*b*) toxic action, corresponding to the ' haptophore ' and the ' zymotoxic ' groups of Ehrlich ; deficiency in the action of complement (or in ' complementing ') does not necessarily imply want of combining affinity, but may be entirely due to the non-sensitiveness of the tissue-molecule to the zymotoxic group.

2. In the case of the three hæmolytic sera studied, the outstanding fact is the large dose both of immune-body and of complement necessary when we use the complement of the same species of animal as that whose corpuscles are being tested.

3. In all three cases there is a relative non-sensitiveness of the corpuscles of the animal to the zymotoxic group of its own complement ; hence a large dose of immune-body is requisite to bring into combination the amount of complement necessary for hæmolysis. In one case (that of the ox) there is also a deficiency in the combining power of the complement with the receptors of the red corpuscles united to immune-body ; from the two conditions acting together complete hæmolysis cannot be obtained.

4. Although differences among the molecules of the same immune-serum may occur, we have found no evidence that the striking differences in the dosage of the immune-body with different complements, and also in the dosage of various complements, are due to the multiplicity of immune-bodies.

No one has yet succeeded in producing an anti-substance or immune-body by injecting an animal with its own corpuscles or cells—such a body as with the aid of complement would produce destruction of these cells. This is manifestly a provision against self-poisoning, and Ehrlich has applied to it the term *autotoxicus horror*. The results which we have brought forward, if they were found to hold

generally, would go to show that even if some substance should appear which acted as an immune-body, there is a further provision whereby the complement of an animal should produce comparatively little harmful effect.

ON THE ACTION OF COMPLEMENT AS AGGLUTININ

In the course of our experiments on hæmolytic sera we have met with the following phenomenon which appears of some interest. It consists in the agglutination of the corpuscles of an animal by its own complement through the medium of the corresponding immune-body derived from another animal, and was observed first in the case of ox's corpuscles, the immune-body used being obtained from the rabbit. The fundamental fact is that if a certain amount of immune-body and ox's complement be added to ox's corpuscles, scarcely any lysis of the corpuscles occurs, but they become agglutinated into large masses which cannot be dissociated by shaking. The immune-serum from the rabbit contains some agglutinin, but the degree of agglutination produced by this is quite trifling compared with that seen when complement also is added. There is thus no doubt that the agglutination phenomenon depends on the co-operation of two substances in a manner comparable to what obtains in lysis. The following are the chief facts regarding the conditions of occurrence of the agglutination and the nature of the agglutinating substance in the ox's serum.

Firstly as regards dosage, a certain amount both of complement and immune-body is necessary. Taking as the standard (one hæmolytic dose) the amount of immune-body sufficient to produce complete lysis of 1 c.c. of 5 per cent. suspension of ox's corpuscles in 0·8 per cent. sodium chloride solution along with guinea-pig's complement, we find that the maximum agglutination is obtained by 3–4 doses of

immune-body and 0·2–0·3 c.c. of ox's serum (complement). The addition of larger amounts of immune-body or of complement has some effect in increasing the agglutination, but only to a trifling extent. It occurred to us that the agglutination might be due to the imperfect lytic action of complement, resulting in the production of an adhesiveness of the corpuscles, but we have found that the agglutination by the complement also occurs when the lysis is complete. If we take some suspension of ox's corpuscles, add several doses (say eight) of immune-body and produce complete lysis by a single dose of guinea-pig's complement, we get a clear fluid, in which nothing can be seen by the naked eye, and in which on microscopic examination the stromata or shadows are seen to be uniformly distributed. If then to such a fluid we add 0·2 c.c. of ox's serum and place the mixture in the incubator for a short time, flocculi appear which are found to be composed of agglutinated stromata. Another example may be given. We have stated (p. 73) that it is not possible to produce complete lysis of ox's corpuscles by ox's complement acting along with the immune-body mentioned, and we have found this always to be the case when the immune-body and the complement are added at the same time. Since making that statement, however, we have observed that if the immune-body is added some time—say an hour—previous to the addition of the complement, then as a rule complete lysis does occur on the addition of the latter, and in such a case there is marked agglutination of the stromata. From these facts it is evident that the agglutination does not depend upon an imperfect lysis.

Another point worthy of note is that the agglutination passes off after some time, usually in five or six hours, at the room temperature. When this occurs the agglutination can be restored on the addition of more complement, whilst additional immune-body is practically without effect. As a rule this second agglutination is not quite so marked as the

first. The passing-off of the agglutination is seen both in the case of the unlysed corpuscles and also in the case of the stromata.

We have also inquired into the temperature at which the agglutination occurs. It is seen within a few minutes (with the doses mentioned above) at 37° C. and in a not much longer time at the room temperature. At 0° C. on the other hand, even after a period of two hours, there is practically no agglutination visible to the naked eye. It would appear from this that the agglutinating action of complement was absent at this temperature, but if we centrifugalize the treated corpuscles it is found that they adhere in masses so firmly that they cannot be separated by shaking. It is thus shown that there is some slight action at 0° C., evidenced by abnormal stickiness of the corpuscles but not by the spontaneous clumping seen at higher temperatures. We therefore cannot say that the agglutinating substance is entirely without action at 0° C., though it is much less marked than at higher temperatures. We may mention another case, viz. guinea-pig's corpuscles, immune-body from the rabbit, and ox's complement, with which combination there is marked agglutination by complement at 0° C., though of course there is no lysis.

When the ox's serum is heated for an hour at 55° C. its agglutinating property, like its hæmolytic, is lost, such a serum having no effect when added to ox's corpuscles treated with immune-body.

From this short statement it is seen that the agglutinating body, studied in the normal serum of the ox, resembles hæmolytic complement both as regards (*a*) its comparative lability—it is rapidly destroyed at 55° C., and (*b*) its acting only in association with immune-body. These circumstances justify the application of ' complement ' to it. It is not quite clear, however, whether this agglutinating complement and the ordinary lytic complement are one and the

same substance. A study of the temperatures at which the two effects—agglutination and lysis—occur, shows that a slight difference exists. This may be due to there being what we may call two complements, or it may simply be due to one substance exerting the agglutinating effect at the lower temperature. Further observations will be necessary on this point. It is also to be noted that the production of adhesiveness of the corpuscles may be manifested before agglutination in the ordinary sense appears.

The facts noted show that the phenomenon of agglutination usually produced by a single body (agglutinin) possessed of combining and agglutinating groups, can also result from the co-operation of two substances in a manner completely analogous to what is seen in bacteriolytic and hæmolytic action. Whether this agglutination of an animal's corpuscles by its own complement may be brought about in conditions of disease by some substance acting like an immune-body remains to be seen. If it does, it is manifest that very grave effects will result.

ON THE FILTRATION OF SERUM COMPLEMENT

A considerable number of facts have been accumulated with regard to the effect of passing organic and other solutions through porcelain filters, though the physical processes underlying such facts are far from being fully understood.

The subject has perhaps been most fully investigated in the case of enzymes, and the literature on the subject has been collected by Levy,[1] to whose paper the reader may be referred for details. It is sufficient to state that various enzymes are kept back to a varying extent by filtering through porcelain and other filters. Levy, for example, found that rennet was completely retained by a Berkefeld filter, that pepsin was partially retained, and that ptyalin and taka diastase passed through. Sirotinin[2] found that when a peptone solution was passed through a porcelain filter, a certain amount was retained at first, but that afterwards the peptone passed through freely. Similar results have also been obtained with metallic colloidal solutions. Zsigmondy[3], for example, quotes the finding of Bredig that a certain amount of colloidal gold is at first retained by a Pukall filter, but that later it passes through freely for a time. He also makes the important observation, that when a certain amount of egg-white is added to the gold solution, the filter becomes permeable, a fact which is of high importance in relation to some of the results obtained below. Observations with regard to the constituents of the serum concerned in hæmolysis and bacteriolysis appear to be comparatively few. Ehrlich and Morgenroth[4], on filtering goat's serum through a Pukall filter, found that the complement concerned in the natural lysis of rabbit's corpuscles was retained, while that acting on guinea-pig's corpuscles passed through. They also found that the normal immune-body

[1] Levy, *Journ. Infect. Diseases*, 1905, ii, p. 1.
[2] Sirotinin, *Zeitschr. f. Hyg.*, 1888, iv, p. 288.
[3] Zsigmondy, *Zur Erkenntnis der Kolloide*, Jena, 1905.
[4] Ehrlich and Morgenroth, *Studies on Immunity*, 1906.

for rabbit's corpuscles passed through the filter. Vedder[1], as a result of filtering fresh serum through a porcelain filter, found that some bacteriolytic complements were retained, e.g. those for the *Bacillus coli* and *Staphylococcus aureus*; whilst others, e.g. that for the typhoid bacillus, passed through. Muir and Ferguson[2] found that when rabbit's corpuscles were lysed with the minimum dose of hæmolytic serum, the surplus of the receptors of the red corpuscles were retained by a porcelain filter, whilst in the case of lysis by water, a very small proportion of the receptors passed through. They also mentioned in connexion with these experiments that immune-body passes through a filter practically unchanged, whilst complement is to a large extent removed from the serum.

The following investigation was undertaken in order to determine certain factors influencing the filtration of serum complement. The experiments were performed with small Berkefeld filters 5·5 cm. in length, and all the results stated below refer to filters of this kind. Some observations were made with Maassen filters, but these were found to be more permeable to complement, although the fluid passed through more slowly than in the case of the Berkefeld filters. Their use was accordingly discontinued. As pointed out by Levy, Berkefeld filters have often a distinctly alkaline reaction, and, accordingly, in all cases before using a filter we have passed water through it till no trace of alkalinity was given by the filtrate. The filters were then dried at 57° C. for twenty-four hours. Fresh serum of normal guinea-pigs was used in all the experiments, and was diluted with an equal volume of 0·85 per cent. solution of sodium chloride before filtration. Comparative experiments were always performed at the same time, and, as nearly as possible, the rate of filtration was uniform; as a rule about 5 c.c. passing through in about two minutes after the filter was saturated with the fluid. As we obtained at first very discordant results when the same filter was used more than once, the explanation

[1] Vedder, *Journ. Med. Research*, 1903, vol. ix, p. 475.
[2] Muir and Ferguson, *Journ. Path. and Bacter.*, 1906, xi, p. 84.

for which will be given later, the observations recorded, except where otherwise stated, were always made with fresh filters. Even under these conditions considerable variability in the action of filters was found, as will be shown below. We always employed a hæmolytic test for estimating the amount of complement, namely, 1 c.c. of a 5 per cent. suspension of ox corpuscles, sensitized with immune-body from the rabbit. The hæmolytic dose was in each case tested before and after filtration of the serum.

Filtration of Hæmolytic Complement

As the result of a large number of experiments, we may state the general conclusion that a small quantity of serum, say 3 to 4 c.c., filtered under the above conditions, loses hæmolytic complement to a great extent, sometimes almost completely. It will be sufficient if we give the two extreme cases which we have met with : (*a*) In one case the dose of complement before filtration was 0·006 c.c., while after filtration 0·4 c.c. gave practically no lysis. We calculated that in this case not more than one seven-hundredth part of the complement originally present had passed through. (*b*) In another case the dose before filtration was 0·003 c.c., whilst after filtration it was 0·025 c.c. In this case the amount of complement had been reduced to a little less than an eighth of the original amount. It is thus seen that great variations in the amount of complement retained occur, and these are also met with when the same serum is filtered through different filters.

With regard to the mechanism by which the complement is retained, we can say nothing further at present than that in all probability it is fixed in some way in the pores of the filters. It might possibly be destroyed in the process of filtration, but we know of no other example of destruction of complement in so short a time. There is, of course, no question as to the complement molecules being mechanically

retained on account of their size. This is made sufficiently evident by the permeability which appears after a time (*vide infra*). In speaking of complement being fixed by the filter, we mean that it apparently combines with its substance, and is thereafter not recoverable. For example, if after filtering fresh serum, some salt solution is passed through the filter, this does not carry the retained complement with it, and if then the filter be pounded in a mortar and treated with salt solution, no complement is obtained in the fluid. We have hitherto failed by any means to recover complement after it has been removed by the filter. As various particulate substances have the property of absorbing complement, we tested the effect of adding the powdered substance of the filter to fresh serum, and shaking the mixture thoroughly. Practically no absorption of complement by the substance of the filter can be shown in this way. Accordingly the passage of the serum through the pores of the filter seems essential to the phenomenon.

The following experiments show that the property possessed by a filter of stopping complement is soon lost in the process of filtration. Such experiments have all been performed on the same plan. A quantity of serum, usually about 4 c.c. diluted with an equal quantity of salt solution, is filtered ; the filter is then washed thoroughly by passing water through it in the reverse direction; it is then thoroughly dried at 57° C., and its properties are again tested by filtering a fresh amount of serum. The following are illustrative examples :—

EXAMPLE 1.—Dose of complement before filtration . 0·005 c.c.
„ „ after filtration . 0·25 „

The filter is then washed and its properties are tested, with the result :—

Dose of complement before filtration . . . . 0·005 c.c.
„ „ after filtration . . . . 0·015 „

It is thus seen that very much more complement, about fifteen times as much, passes through on the second filtration.

EXAMPLE 2.—Dose of complement before filtration . . 0·005 c.c.
„ „ after filtration, about 0·5 „

2nd filtration.—Dose of complement before filtration . 0·005 „
„ „ after filtration . . 0·005 „

In this case the result is even more striking; the filter having been rendered practically permeable to complement. We then tested to what extent the property of retaining complement might be restored by burning the filter at a dull red heat in a hot-air chamber. The filter was the one used in the last example, and after being heated it was tested, with the result :—

Dose of complement before filtration . . . 0·005 c.c.
„ „ after filtration . . . . 0·04 „

It is thus evident that the property of retaining complement has, to a certain extent, been restored by heating the filter.

We have also tested the effect of passing through the filter serum which has been deprived of complement by heating at 55° C. The serum used was that of the rabbit. About 10 c.c. of a mixture of serum diluted with equal parts of salt solution was passed through, then the effect of filtering guinea-pig's complement was tested. The result obtained was—

Dose of complement before filtration . . . 0·003 c.c.
„ „ after filtration . . . . 0·005 „

Accordingly, more than half of the complement has passed through the filter, a result which is never obtained with a fresh filter. Heated serum, that is, serum deprived of complement, thus removes, to a certain extent, the property which a filter has of retaining complement. The effect of passing egg-white through the filter was very much less marked. Egg-white passes through with great difficulty, requiring to be diluted with about ten volumes of salt

solution in order to pass through as quickly as serum. About 10 c.c. of diluted egg-white was passed through, and then the filter was tested as before. Result—

Dose of complement before filtration . . .	0·01 c.c.
,, ,, after filtration	0·15 ,,

Thus only about one-fifteenth part of complement passed through the treated filter, a quantity which is not greater than may sometimes be obtained with a fresh filter.

We have also investigated the effect of temperature on the process of filtration, but have obtained varying results. The earlier experiments seemed to show that complement passed the filter in large amount at 0° C., but in further experiments with fresh filters we found that complement might be retained as effectively at this temperature as at 37 ° C. We can at present make no definite statement on this point.

Effects of Salt Content on Filtration

In the progress of our experiments, the question occurred to us whether there might not be some relation between the retention of complement by the filter and the combining activity of complement. As is well known, the latter does not combine at 0° C. with red corpuscles treated with immune-body, and experiments were carried out to determine the effects of filtration at different temperatures. The results have just been stated. The union of complement with sensitized corpuscles is also prevented by a certain concentration of salt solution, and we accordingly tested whether the process of filtration was influenced by the amount of salt in the fluid. The method used was to add an equal quantity of 10 per cent. sodium chloride solution to fresh guinea-pig's serum, and then to filter the mixture. The filtrate was then diluted with distilled water so as to restore the salt concentration to 0·85 per cent. A quantity of

normal serum with an equal volume of 0·85 per cent. salt solution added to it was filtered at the same time through another filter, and the filtrate was diluted with 0·85 per cent. salt solution, so as to make it of equal bulk to the other. The hæmolytic action of the two fluids was then tested. The following are examples of such experiments, the hæmolytic dose being expressed in terms of the actual amount of serum necessary to bring about complete lysis.

Example 1.—Dose of complement		before filtration . .	0·005 c.c.
,,	,,	filtered in usual way about . . .	0·5 ,,
,,	,,	filtered in 5 per cent. salt solution . .	0·0085 ,,

It is thus seen that while only about one-hundredth part of the complement originally present has passed the filter by the ordinary method of filtration, more than half the complement has passed the filter in the presence of 5 per cent. sodium chloride.

Example 2.—Dose of complement		before filtration . .	0·01 c.c.
,,	,,	filtered in usual way about . . .	0·13 ,,
,,	,,	filtered in 5 per cent. salt solution . .	0·01 ,,

In this case practically all the complement in the salted serum has passed.

Example 3.—Dose of complement		before filtration . .	0·006 c.c.
,,	,,	filtered in usual way .	0·2 ,,
,,	,,	filtered in 5 per cent. salt solution . .	0·013 ,,

In this case, fully a half of the complement in the salted serum passed the filter.

It is thus evident that the addition of an amount of salt sufficient to inhibit the hæmolytic action of complement prevents in some way, which we cannot at present explain, the retention of complement in the pores of the filter.

On the Filtration of Complement along with Immune-body

As already noted (p. 91), we found that immune-body passes through the filter practically unchanged, and our results in the present series of experiments are confirmatory. We accordingly considered it a matter of interest to inquire how the filtration of immune-body was affected by the presence of complement. As is well known, complement and immune-body when they are present in a mixture are readily separable from each other by absorption methods at 0° C., but Ehrlich has suggested that they probably unite at a higher temperature, the supposed amboceptor action of the immune-body coming into play. Now if this were so, we should expect that the immune-body which had combined with complement would be retained by the filter along with the latter, and thus the amount of immune-body in the filtrate would be diminished, as compared with the amount when immune-body is filtered alone. In every case, however, we obtained the result that even when complement is present, immune-body is practically unaffected by filtration.

In the two first experiments we have as nearly as possible used corresponding amounts of immune-body and complement, as shown by their hæmolytic doses. In the third experiment a considerable excess of complement was present, as we supposed that possibly by this means there might be retention of immune-body. The complement (fresh serum of guinea-pig) and the immune-body (serum of a rabbit treated with ox's corpuscles, deprived of complement by heating at 55° C.) were mixed together in the quantities indicated, and the volume was made up to 8 c.c. with 0·85 per cent. sodium chloride solution. The mixture was then filtered as described.

Example 1.—One hundred doses of complement (hæmolytic dose,

0·005 c.c.) and one hundred doses of immune body (hæmolytic dose, 0·003 c.c.). Filtration at 37° C.

Dose of complement before filtration . . .	0·005 c.c.
,, ,, after filtration	0·15 ,,
Dose of immune-body before filtration . . .	0·003 ,,
,, ,, after filtration . . .	0·003 ,,

Example 2.—Four hundred doses of complement and four hundred doses of immune-body: incubated for one and a half hours at 37° C. and then filtered.

Dose of complement before filtration . . .	0·01 c.c.
,, ,, after filtration, about . .	0·4 ,,
Dose of immune-body before filtration . . .	0·0008 ,,
,, ,, after filtration . . .	0·0008 ,,

Example 3.—One thousand doses of complement and four hundred doses of immune-body filtered after being allowed to stand for two hours at room temperature.

Dose of complement before filtration . . .	0·005 c.c.
,, ,, after filtration . . .	0·125 ,,
Dose of immune-body before filtration . . .	0·0025 ,,
,, ,, after filtration . . .	0·0025 ,,

In all these examples it is seen that the immune-body passes through the filter unchanged in amount, even when it is associated with a large quantity of complement. The complement, on the other hand, undergoes a marked reduction, just as in the previous experiments. Accordingly, so far as this method of investigation shows, there is no indication that immune-body unites with complement at the temperatures at which complement is known to act, namely, room temperature and especially at 37° C. It might be suggested that complement unites with immune-body, but that the union is of such a loose nature that the two substances are separated in the process of filtration. This, however, appears rather an extravagant supposition, and is one for which we have no evidence.

Summary of Results

1. In the early stages of filtration of normal serum through a Berkefeld filter complement is in great part, sometimes almost completely, removed from the serum, and the complement retained by the filter is not recoverable.

2. In the process of filtration the filter soon becomes permeable to complement. Permeability can also be produced by the passage of serum previously deprived of complement by heating.

3. Complement rendered inactive by the addition of 5 per cent. sodium chloride passes through a Berkefeld filter, the original value of complement being almost completely regained on making the necessary dilution of the filtered salted serum.

4. Immune-body passes through the filter practically unchanged. It passes equally well when it is mixed with a corresponding quantity of complement at 37° C. and filtered at this temperature, while the complement is retained. Such filtration experiments supply no evidence that complement and immune-body unite at this temperature.

SECTION C. ON THE PROPERTIES OF ANTI-IMMUNE-BODIES

Of papers published within recent years which concern the properties of immune-sera and bear upon the general theory of immunity, one of the most important is that of Bordet[1] on *antisensibilisatrices* (anti-immune-bodies, anti-amboceptors). This observer obtained an anti-immune-body by injecting the serum of a normal animal (rabbit) into an animal of another species (guinea-pig), and found that there was developed an anti-immune-body which had the property of neutralizing the various immune-bodies which might be developed by active immunization of the first animal. In this way a certain community as regards combining properties was demonstrated among the immune-bodies of a given species of animal. He studied the properties of the anti-immune-body, and in particular showed that it did not combine with the cytophile group of the immune-body, and therefore did not prevent the usual combination of the cell-receptor with the immune-body. This fact he held to be inconsistent with Ehrlich's views regarding the amboceptor constitution of immune-body. His observations on the neutralizing effect of the anti-immune-body were carried out by means of hæmolytic sera. Ehrlich and Sachs[2] confirmed the chief results obtained by Bordet, but so far from admitting the establishment of any objection to the amboceptor theory, claimed that they supplied strong evidence in support of it. They maintained, in fact, that the anti-immune-body acted by combining with the complementophile group of the amboceptor, and thus prevented the union of complement,

[1] Bordet, *Ann. de l'Inst. Pasteur*, 1904, Tome xviii, p. 593.

[2] Ehrlich and Sachs, *Berlin. klin. Wochenschr.*, 1905, pp. 557, 609.

stating also that the results showed that the various immune-bodies from the same species had a similar complementophile apparatus. The thoretical bearings of the facts established, will, however, be discussed later.

As the subject appeared to us to be of high importance, we have repeated the various experiments, and have in addition studied the combining properties of anti-immune-bodies by quantitative methods. We have especially inquired whether the combination of anti-immune-body can thus be demonstrated even when its action in preventing lysis of red corpuscles is not apparent, and have found this to be the case. In this connection we have been forced to consider certain circumstances which influence the occurrence of lysis, and have carried out observations on the effect produced by altering the medium of suspension of the red corpuscles. The investigation thus consisted of two main parts, viz. (1) a preliminary part, dealing with the dosage of complement in different media, which has been recorded above (p. 62), and (2) a part dealing with the main subject of the investigation, viz. anti-immune-bodies.

The methods employed are those described in previous sections and need not be repeated. The test quantity of corpuscles generally used was half the usual amount, viz. 0·5 c.c. of a 5 per cent. suspension of washed corpuscles in 0·85 per cent. salt solution. The anti-immune-body was obtained by injecting guinea-pigs with the serum of the normal rabbit, a guinea-pig of about 500 grams receiving two intraperitoneal injections of from 4 to 6 c.c. on two occasions, with an interval of ten days between, and then being killed about ten days after the last injection. Several guinea-pigs were thus treated at the same time, and the various sera were mixed together and heated at 55° C. to deprive them of complement. The immune-body in most cases is that obtained by injecting rabbits with ox's corpuscles freed from serum by washing in salt solution. In some experiments we also used an anti-

immune-body to the guinea-pig's immune-bodies, which was obtained by injecting a rabbit with guinea-pig's serum. It is convenient, as Bordet has done, to indicate that a serum has been deprived of the action of complement by heating, to place 55° C. after its name, e.g. guinea-pig's serum 55° means the serum of a normal guinea-pig, heated for an hour at 55° C.

Demonstrations of the Action of Anti-immune-body

As stated above, the anti-immune-body is obtained by injecting the guinea-pig with the normal serum of the rabbit. We shall speak of such a serum obtained from a guinea-pig as anti-IB; it is, of course, deprived of complement by heating at 55° C.

The fundamental result obtained by Bordet, showing that the anti-immune-body unites with red corpuscles sensitized with immune-body and protects them against the action of complement, is readily demonstrated. The following may be taken as an example.

The experiment may be represented thus :—

	W	*W*	
A. RCs + 3 IB	+ 0·3 c.c. anti-IB	+ C	no lysis [1]
B. RCs + 3 IB	+ 0·3 c.c. guinea-pig's serum 55°	+ C	lysis [1]

The vertical interrupted line represents a period of incubation at 37° C., for an hour unless the time is stated.

W signifies that the corpuscles are washed and centrifugalized after incubation.

Two series of tubes (A and B) each containing 0·5 c.c. of suspension of red corpuscles are prepared, and to each three hæmolytic doses of immune-body are added. After an hour to allow for combination the corpuscles are centrifugalized and washed with salt solution (all the immune-body present is therefore in combination with the red

[1] The corpuscles are suspended in guinea-pig's serum 55° C.

corpuscles). To each of the tubes in one series (A) we add 0·3 c.c. of anti-immune-body; to the tubes in series B, 0·3 c.c. of normal guinea-pig's serum heated at 55° C. as a control. The tubes are placed in the incubator at 37° C. and after an hour they are centrifugalized and the contents washed with salt solution. They are again centrifugalized and the salt solution is pipetted off. To each tube is added 0·5 c.c. of guinea-pig's serum heated at 55° C. We have thus two series of tubes, in one of which the corpuscles are combined with IB and anti-IB, in the other only with IB. We then test the effects of complement by adding varying amounts to the several tubes. It is found that in series A 0·1 c.c. of complement produces practically no lysis, whilst in series B the same amount produces complete lysis. In series A even 0·2 c.c. of complement produces only slight lysis.

On the Mode of Action of Anti-immune-body

Does the anti-immune-body act by *preventing the union of complement* with the red corpuscles treated with immune-body or by in some way *inhibiting the toxic action* of complement? The former will be shown to be the case, if we separate the fluid from a tube in which lysis has been prevented by anti-immune-body and find that it contains uncombined complement, the test being made in the usual way by adding red corpuscles treated with immune-body and observing whether lysis results. Experiments carried out on these lines show clearly that in every case where the anti-immune-body prevents lysis, complement has been kept out of combination. This is in confirmation of the result arrived at by Bordet. In our initial experiments the red corpuscles were suspended in guinea-pig's serum 55°, in accordance with Bordet's method, but we afterwards used salt solution as the medium of suspension and investigated the action of the anti-immune-body in general, even where lysis

is not prevented. The details of method are of similar nature in the two cases and are given below.

Bordet found that the action of anti-immune-body could be demonstrated when the corpuscles were suspended in guinea-pig's serum 55° C., whereas this result was usually not obtained when they were suspended in salt solution. *A priori* it is unlikely that the action of anti-immune-body is not identical in the two conditions, and we have investigated whether this is the case. We find that in salt solution the effect of anti-IB can be demonstrated in two ways: (*a*) by its delaying lysis, and (*b*) by its keeping out complement. We proceed as before by adding immune-body and anti-immune-body to the one series of tubes containing 0·5 c.c. suspension of red corpuscles, and to the other series immune-body and normal guinea-pig's serum heated at 55° C. After the corpuscles have been washed, complement is added in varying amounts.

A. Red corpuscles + 5 doses of IB + 0·25 c.c. anti-IB.
Amounts of guinea-pig's C 0·01, 0·015, 0·02, 0·025, 0·03, 0·04, 0·05, 0·06 c.c.
Dose of C = 0·005 c.c.
Amount of C taken up in 1½ hours at 37° C. = 0·012 c.c.

B. Red corpuscles + 5 doses of IB + 0·25 c.c. guinea-pig's serum 55° C.
Amounts of C added as in A.
Amount of C taken up = 0·023 c.c.

The difference in the amount of complement taken up in the two series is thus very striking, being 0·011 c.c. or more than two hæmolytic doses of C, this amount being kept out by the action of anti-IB.

The action of the anti-IB was also shown by the comparative rapidity of the initial lysis in the two series. After twenty-five minutes, lysis in series A was just complete in the tube containing 0·04 c.c. of complement, whilst at the same

time in series B it was complete in the tube containing ·015 c.c. of complement; at the end of forty minutes, however, lysis was complete in all the tubes of both series. The action of anti-IB can thus be demonstrated quite clearly by the rate of occurrence of lysis when the corpuscles are suspended in salt solution, even though lysis is not prevented. The difference as regards lysis in the two media is due to the fact that a much larger dose of complement is necessary when the corpuscles are suspended in guinea-pig's serum 55°; that is, complement acts more feebly, and any further diminution in its action is much more apparent. We therefore conclude that whether lysis is prevented or not, *the anti-immune-body in every case keeps a certain amount of complement out of combination.*

Action of Anti-immune-body on Multiple Immune-bodies developed by Active Immunization

Bordet found that anti-immune-body acted on all the immune-bodies supplied by the animal whose serum was injected in order to produce the anti-immune-body. In addition to the immune-body for ox's corpuscles we have also investigated by quantitative methods the action of anti-immune-body in the case of the immune-body for guinea-pig's corpuscles (obtained by injecting a rabbit with these corpuscles).

Example. Dose of IB for guinea-pig's corpuscles = 0·006 c.c.[1]

Two series of tubes, A and B, containing 0·5 c.c. suspension of red corpuscles and four doses of immune-body, are prepared as before, those in A also receiving 0·2 c.c. anti-IB—the details need not be repeated. In series A the amount of guinea-pig's complement taken up was 0·015 c.c.; in series B, 0·045 c.c. Therefore the anti-immune-body (0·2 c.c.)

[1] i.e. along with rabbit's complement. On variations in dosage *vide* p. 72.

kept out of combination 0·03 c.c. of C. (The test for uncombined complement was ox's corpuscles treated with immune-body.)

It is interesting to note that in a similar experiment made at the same time with ox's corpuscles treated with four doses of immune-body the amount of complement kept out by 0·2 c.c. of anti-immune-body was the same as in the case of guinea-pig's corpuscles, i. e. 0·03 c.c. of complement, as tested with corpuscles treated with immune-body. In other words, apparently the same amount of anti-immune-body had been taken up by the two kinds of corpuscles treated with their corresponding immune-body.

Action of Anti-immune-body on Natural Immune-bodies

We have investigated this in the case of the anti-immune-body obtained by injecting the rabbit with guinea-pig's serum—the anti-immune-body thus neutralizing the immune-bodies derived from the guinea-pig. The normal serum of the guinea-pig has a varying degree of hæmolytic action on both rabbit's and ox's corpuscles, and this, as was shown by Ehrlich, is due to the combined action of natural immune-bodies with complement. By placing the corpuscles (rabbit's or ox's) in contact with the guinea-pig's serum at 0° C. a certain proportion of immune-body enters into combination, as is shown by the fact that when the corpuscles are washed in salt solution and complement is added a certain amount of lysis takes place. The method of testing the action of anti-immune-body is thus the following. The corpuscles are placed in the serum of a normal guinea-pig for an hour at 0° C. and are then washed free of serum; they have thus taken up natural immune-body from guinea-pig's serum. They are then divided into two sets, to one of which is added 0·2 c.c. of anti-immune-body from the rabbit, and to the other, as a control, 0·2 c.c. of rabbit's

serum 55°. After an hour at 37° C. the corpuscles are washed and the same amount of complement is added to each. It is found that the resulting lysis is less in the case of the corpuscles treated with anti-immune-body. The difference is marked in the case of the ox's corpuscles; slight, though distinct, in the case of the rabbit's corpuscles. Anti-immune-body thus apparently acts in the same way on natural immune-bodies as on those produced by artificial immunization. This is additional evidence, if such were needed, that the immune-body naturally present in a serum has the same constitution as that developed by active immunization.

(*Note.* The 'complement' used in these experiments was guinea-pig's serum, which can be practically freed of the natural immune-body for ox's corpuscles by contact with the corpuscles at 0° C. In the case of the immune-body for rabbit's corpuscles, however, so complete a result is not obtained, though a certain amount of immune-body is taken up. Thus the serum cannot be completely freed of its natural hæmolytic action. As there is, however, much more complement relatively than natural immune-body, the action of anti-immune-body can quite well be demonstrated.)

Relation of Anti-immune-body to the Combination of Immune-body with Receptors

We have seen that the anti-immune-body acts by preventing the combination of complement with R + IB molecules. We have, however, considered it advisable to investigate by quantitative methods whether it has any action in preventing the union of immune-body with the receptors of the red corpuscles directly. The principle is to test whether the same amount of immune-body is taken up by the red corpuscles in the presence of anti-immune-body as when it is absent. This is best done by observing the saturation point of the red corpuscles with immune-body in the two conditions; that is, to find how many doses of immune-body must be added before one dose remains free.

Two series (A and B) of seven tubes, each containing ·5 c.c. of salt solution, are taken :—

1. To each tube in A 4 doses of IB and 0·2 c.c. anti-IB (from guinea-pig).

To each tube in B 4 doses of IB and 0·2 c.c. guinea-pig's serum 55° (as a control).

They are placed in the incubator for an hour at 37° C.

2. 0·5 c.c. of suspension of red corpuscles is added to each, and the tubes are again incubated for an hour.

The contents of each tube are then washed and centrifugalized. The corpuscles in all the tubes have thus been treated with the same amount of IB, those in series A being treated with anti-IB also. We have thus to test how much IB will be taken up in the two series.

3. To the several tubes in the two series 1, 2, 3, 4, 5, &c., doses of IB are added.

4. After an hour the tubes are centrifugalized and the separated fluid from each is added to red corpuscles along with a sufficient amount of complement, in order to show the amount of free IB in each.

The result is that lysis is the same in both series, being first complete in the fifth tube in each. As originally four doses of IB were added and then five to this tube, this means that whether anti-IB is present or not the same amount of IB is taken up—when nine doses of IB are added, one dose remains free.

We therefore conclude that *the anti-immune-body in question has no effect in interfering with the combination of immune-body with the receptors of the red corpuscles.* If the anti-immune-body had prevented the union of immune-body with the receptors of the red corpuscles, then we should have found that in the series treated with anti-immune-body more immune-body would have to be added subsequently before one dose remained free than was necessary in the series treated with immune-body alone.

On the Combination of Natural Immune-bodies with Anti-immune Body

In view of the general law that an anti-substance developed in an animal combines with the substance introduced, and in view of the fact that the serum of the normal rabbit is used for injection, we would expect that bodies in this serum would unite with the anti-immune-body developed as the result of the injection. Bordet surmised that the anti-immune-body developed in the guinea-pig was the result of the introduction of the natural immune-bodies in the rabbit's serum, of which doubtless there is a large number, and Ehrlich and Sachs are decidedly of the same opinion. The combining properties of these immune-bodies might theoretically be demonstrated in two ways, viz. (*a*) by allowing them to combine with the anti-immune-body and thus interfering with its ordinary action, and (*b*) by allowing them to act on the anti-immune-body after it has combined with the red corpuscles treated with immune-body, and removing in part its inhibitory action. Both methods have been carried out.

(*a*) The direct combination of the anti-IB from the guinea-pig with the natural immune-bodies in rabbit's serum. The following is the scheme of experiment :—

			W
A.	Anti-IB + rabbit's serum 55° (natural IBs)	+ $\frac{\text{RCs} + 5\,\text{IB}}{\text{washed}}$	Estimate how much C can then be taken up.
B (control).	Anti-IB + guinea-pig's serum 55°	+ $\frac{\text{RCs} + 5\,\text{IB}}{\text{washed}}$	Do. do.

Example. In A the amount of C taken up was 0·014 c.c.
In B ,, ,, ,, ,, ,, 0·009 c.c.

As half tubes (0·5 c.c. of suspension of red corpuscles) were used, and the dose of C for half a tube was 0·0037, there is a difference of fully a dose of C in the two series, i. e. the natural immune-bodies have united with an amount of anti-immune-body which would keep out more than a dose of C.

Other experiments in which ox's serum 55° and simple salt solution were used in the control instead of guinea-pig's serum 55°, gave the same results. When rabbit's serum 55° is used, the diminished action of anti-immune-body (owing to its combination with the natural immune-bodies) is also shown by the much greater rapidity of lysis than is the case in the control.

We therefore see that *of the three heated sera tested the rabbit's is the only one which contains molecules capable of combining directly with anti-immune-body and thus of diminishing its action.*

The experiment quoted as an example was the one which showed most prominently the direct combination of the natural immune-bodies with anti-immune-body ; in certain others it was practically imperceptible, though its influence on the rapidity of lysis was quite apparent. On the whole the results of the action of the natural immune-bodies in dissociating the anti-immune-body, now to be recorded, are more striking. The reason of this will be discussed below.

The Dissociation of Anti-immune-body by the Corresponding Natural Immune-bodies

Bordet found that the normal rabbit's serum 55° (containing natural immune-bodies) had the power of separating anti-immune-body after it had combined with red corpuscles treated with immune-body. We have obtained in the case of our anti-immune-body results similar to those of Bordet and of striking character. The principle of the method of demonstration is simply this :—we take two series of tubes containing the same amounts of red corpuscles, of IB and of anti-IB ; and after time for combination is allowed the corpuscles are centrifugalized and washed. To those of one series normal rabbit's serum 55° is added ; to those of the other series, guinea-pig's serum 55°, as a control. After centri-

fugalizing and washing the corpuscles we test how much complement can be taken up by the corpuscles in the two series. The following is the scheme :—

		W	*W*	*W*
A.	$\overline{\text{RCs} + 5\,\text{IB}}$	+ anti-IB	+ 0·3 c.c. rabbit's serum 55°	Find how much C is taken up.
B (control).	$\overline{\text{RCs} + 5\,\text{IB}}$	+ anti-IB	+ 0·3 c.c. guinea-pig's serum 55°	Do. do.

The average result may be said to be that treatment with 0·3 c.c. rabbit's serum 55° in A had the effect of displacing sufficient anti-IB to allow the combination of fully two doses of complement more than in B.

In the control we have in other experiments replaced the guinea-pig's serum 55° by ox's serum 55° and also by salt solution. In all the experiments the same result has been obtained, viz. the treatment with rabbit's serum 55° *increases the power of taking up complement*, i. e. turns anti-IB (for rabbit's IB) out of combination with $\overline{\text{RCs} + \text{IB}}$.

The dissociation of anti-immune-body by the natural immune-bodies is thus amply proved; and, as we stated above, the result is more striking than that supplied by the experiment showing the prevention of combination of anti-immune-body by the natural immune-bodies. We cannot say definitely why this should be the case ; but a possible explanation might be offered by supposing that there was an excess of anti-immune-body in the *preventive* experiment, so that a portion of it was free to combine with the red corpuscles united with immune-body (RCs + IB) and thus its effect was only slightly diminished. On the other hand, in the *dissociation* experiment 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 a considerable quantity. Although we have not tested this hypothesis by varying the amounts of anti-immune-body,

the results of the various experiments both on the prevention of union of anti-immune-body and also on its dissociation, are quite in accord in the two series.

On the Possibility of Preventing the Combination of Anti-immune-body by Means of Complement

This is, of course, the converse to the experiments recorded above with regard to the keeping out of complement by means of anti-immune-body. Our experiments were carried out by heating two equal quantities (A and B) of red corpuscles at 55° C. over night as described above, then adding to both the same amounts of immune-body, whilst to those in A a large amount of complement is also added. After time for combination the corpuscles were washed and the fluid pipetted off. The two sets of corpuscles were then added to the same amounts of anti-immune-body, and then the amount of anti-immune-body in the supernatant fluid was tested for in the usual way. The results of several experiments all agreed in showing no distinct difference in the amounts of anti-immune-body taken up. We were thus unable to show that complement kept out anti-immune-body—a result which would have been practically conclusive as to the union of these two substances with the same combining-group.

As stated in the introduction—Bordet considered that the results which he obtained with regard to anti-immune-bodies, and which in all important points we have been able to confirm, were inconsistent with Ehrlich's side-chain theory, and especially with his view regarding the amboceptor constitution of immune-bodies. His criticism, however, is based on the assumption that an anti-immune-body must combine with the cytophile group of the amboceptor, that is, must have the same combining-group as the corresponding cell-receptor. The facts demonstrated of course show that this

is not the case. The combination of anti-immune-body with immune-body in no way interferes with the combination of the latter with the cell-receptor, and, as Bordet himself showed, the anti-immune-body acts by preventing the union of complement. As Ehrlich and Sachs state, however, the objections brought forward by Bordet entirely lose their force if it is maintained that the anti-immune-body unites with the complementophile group of immune-body. They hold that the immune-bodies of a given species have the same complementophile apparatus, and that it is a matter of indifference which immune-body is used to produce the anti-immune-body. The latter will act on all the immune-bodies with the same complementophile groups. It must be granted that the facts recorded with regard to anti-immune-bodies appear in this respect to support Ehrlich's hypothesis ; whilst Bordet offers no theoretical explanation of the facts demonstrated. What is clearly established is that the anti-immune-body unites with a combining-group of the immune-body which is not the cytophile group, and that this union prevents the union of complement with the complex immune-body + receptor. From the point of view of the animal injected with immune-bodies, the development of the anti-immune-body may be regarded as a mechanism for preventing the associated action of the immune-bodies and its complement (though the action of other complements is also annulled).

This may be due to the anti-immune-body occupying the complementophile group of the immune-bodies ; but that is not absolutely proved. When we consider the combining relationships of the different substances certain difficulties arise which have not yet received an explanation. As shown in a former paper, rabbit's and guinea-pig's complements combine with the same group in lysis by means of an immune-body from the rabbit, each keeping out the other from combination. The same holds with regard to an immune-

body from the guinea-pig. According to the lock and key analogy, the complementophile groups of the immune-bodies of the rabbit and the guinea-pig so far as they are satisfied by the same complements would thus appear to be the same. As, however, the anti-immune-body to the rabbit's immune-body does not act on the guinea-pig's immune-bodies, the complementophile groups would appear to be different. It may also be remarked that a somewhat similar contradiction apparently existed in the anti-complement to the rabbit's complement not acting on guinea-pig's complement, while both complements showed the same combining affinities in lysis, i. e. in one instance they had apparently different combining-groups, while in the other the groups were similar. In this case, however, the apparent contradiction has been explained by the facts established regarding deviation of complement (p. 133). In the case of anti-immune-bodies the difficulty might possibly be explained by the different energy of combination in the two instances, the anti-immune-body having a comparatively weak affinity for rabbit's immune-body (as is shown by the experiments) and no affinity for guinea-pig's immune-body, whilst guinea-pig's and rabbit's complement, owing to their strong affinity, will combine with either guinea-pig's or rabbit's immune-body; that is, qualitative differences come out in the case of weak combination which are not seen in the case of combination of a powerful nature. Against such a view, however, is the fact that we have failed to find any evidence that complement prevents the combination of anti-immune-body with immune-body—a result which would point to the complement and anti-immune-body uniting with different combining-groups. At present, however, no definite conclusion can be drawn. All that we can say is that anti-immune-body annuls the usual result of the union of immune-body with receptor, that is, prevents the combination of complement.

Summary of Results obtained by Hæmolytic Methods

1. The anti-immune-body obtained by injecting the normal serum of the rabbit has been shown to act on two immune-bodies produced in the rabbit by active immunization, and the corresponding anti-immune-body obtained by injecting guinea-pig's serum was shown to act on two natural immune-bodies in the serum of that animal.

2. The anti-immune-body is shown by quantitative experiments not to interfere in any way with the combination of immune-body with the receptors of the red corpuscles.

3. The anti-immune-body combines with red corpuscles treated with immune-body (sensitized corpuscles) and interferes with the combination of complement. Lysis may thus be prevented by the anti-immune-body; but where this is not the case it is shown by quantitative methods that a certain amount of complement is kept out of combination.

4. The natural immune-bodies of the serum injected combine directly with the anti-immune-body developed, and have also the power of dissociating anti-immune-body after it has combined with immune-body fixed to red corpuscles. The combination of anti-immune-body is a comparatively weak one and belongs to the group of reversible actions.

5. We have been unable so far to demonstrate that the previous combination of complement prevents the union of anti-immune-body with red corpuscles treated with immune-body.

SECTION D. ON THE HÆMOLYTIC RECEPTORS OF THE RED CORPUSCLES

The molecules in the red corpuscles with which an immune-body combines are generally known by the name of ' receptors ', applied to them by Ehrlich,—they may be called hæmolytic receptors, to distinguish them from other molecules of the same class. We may also put the matter in another form, and say that the receptors on injection give rise to the production of anti-receptors. Further, the combination receptor *plus* anti-receptor has the power of taking up complement, hæmolysis resulting from this union. In the following research we have dealt with certain questions as to the properties of the molecules which act as receptors in hæmolysis and as antigens for immune-bodies when the red corpuscles of an animal are injected into another of different species. The question as to what part of the red corpuscle contains the receptors has been the subject of a certain number of investigations, of which a short *résumé* may be given.

V. Dungern [1] (1899) found that after lysis was produced by laking agents the receptors were detectable neither in the stromata nor in the hæmoglobin-containing fluid, and he concluded that the receptors were labile bodies, and were destroyed in the process of laking. He used the corpuscles of the goose, and the injections were made into rabbits. Lysis was produced by water and ether, and the stromata were then dissolved in 5 per cent. magnesium sulphate, or in 1 per cent. hydrochloric acid; the molecules might therefore have undergone important chemical changes. Bordet [2] (1900) found that the stromata had the power of fixing immune-body and thereafter of fixing complement, and that the injection of stromata produced a hæmolytic serum; on the contrary, only negative results were obtained when the clear fluid, obtained by centrifugalization after

[1] V. Dungern, *München. med. Woch.*, 1899, p. 449.
[2] Bordet, *Annales de l'Inst. Pasteur*, 1900, vol. xiv, p. 257.

lysis, was used. His results, therefore, go to show that the receptors remain attached to the stromata. He used the corpuscles of the rabbit and the corresponding immune-body obtained from the guinea-pig. The latter animal was also employed for testing the result of the injection of stromata. Nolf[1] (1900), on the other hand, arrived at a conclusion quite contrary to that of Bordet. He employed the corpuscles of the fowl, and the injections were made into the rabbit. He produced lysis with water, then made up with sodium chloride, and separated the stromata from the fluid by centrifugalization. He found by this means that the injection of the stromata gave rise to agglutinin but little lysin, whilst the cellular contents (*contenu globulaire*), which were dissolved out into the fluid, had the converse property, giving rise to much lysin but little agglutinin. According to his results, most of the hæmolytic receptors are separated from the stromata on lysis with water. G. N. Stewart[2] (1904) arrives at results which to a certain extent resemble those of Nolf, inasmuch as they point to a closer relation between the agglutinogenic property and the stromata than between the lysinogenic property and the stromata; he considers, however, that Nolf's decided conclusions are not justified. He finds that both the stromata and the hæmoglobin-containing fluid obtained after lysis have the property of giving rise to agglutinins and lysins on injection, but that the agglutinating property is more marked when the stromata are injected, the hæmolytic property when the separated fluid is used. When milder hæmolytic agents are used, there is less separation of the agglutinogenic receptors from the stromata than when water is used. In most of his experiments he used rabbits' corpuscles, and the injections were made into guinea-pigs; the observations are thus comparable with those of Bordet, and the variance in the results is noteworthy.

From this *résumé* it will be seen that the results obtained by the different observers are of conflicting nature, and it cannot be considered satisfactorily determined whether the receptors of the red corpuscles pass into the surrounding fluid or remain attached to the stromata when the corpuscles are lysed, say with water. All the observers mentioned

[1] Nolf, *Annales de l'Inst. Pasteur*, 1900, vol. xiv, p. 297.
[2] G. N. Stewart, *Amer. Journ. Physiol.*, Boston, 1904, vol. xi, p. 250.

employed the method of injecting the fluid and stromata respectively into animals, and observing whether or not immune-body was developed. Bordet alone obtained confirmatory results by test-tube experiments, though he does not give details as to the exact amounts. It has seemed to us desirable to investigate the question in this latter way, as the experiments can be much more rapidly and exactly carried out. And, further, if the method of animal injection is to be employed, the blood of the species of animal injected ought normally to have no hæmolytic action on the corpuscles used. The serum of the guinea-pig produces a certain amount of lysis of rabbit's corpuscles, and this varies much in the case of different animals: there would accordingly be great liability to error unless the hæmolytic action present after injection of stromata or fluid were of pronounced character. It has now been demonstrated so frequently that when a substance injected into the body of an animal gives rise to the production of an anti-substance there is a specific combining affinity between the substance and the anti-substance, that this may be accepted as a general law. In the case before us the question thus comes to be, does the immune-body combine with molecules in the 'stromata' or in the 'contained fluid' of the red corpuscles; or, in other words, when the corpuscles undergo lysis, do the receptors remain in the stromata or do they pass out into the surrounding fluid? It might appear that an answer to this question could be obtained by adding immune-body to the stromata and separated fluid respectively, and observing whether or not it was taken up. As, however, it has been shown that the combination of immune-body with receptors is a very loose one (*vide* p. 12)—in fact, belongs to the class of 'reversible actions'—it follows that even if immune-body did enter into combination, a certain amount would be obtainable by dissociation, and thus accurate knowledge as to the combination or non-

combination of immune-body would not be obtained. On the other hand, we have demonstrated that the combination of complement with the receptors of the red corpuscles through the medium of immune-body is a very firm one, and thus a test for the presence of receptors is afforded. To put the matter briefly, we may say that if the receptors are present in a solution or in a suspension, and if complement be added along with immune-body, a certain amount of complement will be taken up in firm combination, so that fresh red corpuscles treated with immune-body do not undergo lysis when they are added to the mixture.[1] The test, then, for the presence of the receptors in question consists in adding complement and immune-body and observing whether or not the former is diminished in amount. As hæmolytic agents we have chiefly used sterile water and a hæmolytic serum, though some experiments have been also performed with ether. With regard to hæmolytic sera it may be again noted that in many instances red corpuscles can take up several hæmolytic doses of immune-body, or, in other words, there are many more receptors than are necessary for complete hæmolysis. Therefore, if we add multiple doses of immune-body and produce lysis with a single dose of complement, there remain many receptors combined with immune-body whose affinity for complement is still unsatisfied. We can test whether these molecules are in the stromata or free in the fluid. In all the experiments we have used ox corpuscles, the corresponding *immune-body* being obtained from rabbits treated with injections of ox's corpuscles (washed in salt solution), while the *complement* is normal guinea-pig's serum from which the natural immune-body for ox's corpuscles has been removed by contact with these corpuscles for an hour at 0° C.

[1] This is merely an example of the deviation of complement by an antigen *plus* its anti-substance, a reaction which has come into general application since the above was written. See also Part II (p. 133).

Effects of Laking Agents on the Receptors

In the case of such a relatively disruptive hæmolytic agent as water it is necessary as a preliminary to inquire whether it has any destructive effect on the receptors of the red corpuscles. Such an inquiry is carried out by taking a given amount of red corpuscles, adding water to produce lysis, and then making up with concentrated sodium chloride solution so as to obtain a concentration of 0·85 per cent. We then test whether such a mixture has the property of taking up complement through the medium of immune-body to the same extent as a suspension of the same amount of red corpuscles which have not been lysed. The following are the details :—

10 c.c. of a 5 per cent. suspension of red corpuscles in 0·85 per cent. sodium chloride solution are taken and centrifugalized. After the supernatant fluid has been pipetted off, complete lysis of the sedimented corpuscles is produced by adding sterile water. The mixture is left for half an hour, and thereafter 1 c.c. of an 8·5 per cent. sodium chloride solution is added and the volume is made up to 10 c.c. by the addition of water. The mixture is then thoroughly shaken, and 1 c.c. is placed in each of a series of tubes. To form a control series, we place 1 c.c. of the original suspension of corpuscles in each of a similar number of tubes.

We have thus two series of tubes, each tube in one series containing a given amount of red corpuscles, each tube in the other containing the same amount of corpuscles lysed with water. To each tube in the two series we add several hæmolytic doses of immune-body, and then to the tubes in each series increasing amounts of complement. The tubes are then placed in the incubator for two hours at 37° C., and the presence of complement is tested for by adding to each 1 c.c. of suspension of red corpuscles treated with immune-body.

Example.—The above procedure is carried out. Eight doses of

immune-body are added to each tube, and then to the tubes in the two series 0·075, 0·1, 0·125, 0·15, 0·175, 0·2 and 0·25 c.c. of complement. The tubes in both series give the same amount of complement taken up, the tubes with 0·15 c.c. of complement giving a mere trace of lysis of the added corpuscles. Therefore about 0·145 c.c. of complement was taken up in each. M.H.D. of complement = 0·015 c.c.

The receptors are accordingly not destroyed by lysis with water.

It is evident that various hæmolytic agents might be tested in the same way. The only other one which we have used is ether.

In this case a sufficient amount of ether to produce complete lysis is added to the suspension of red corpuscles (say 2 c.c. of ether to 10 c.c. of suspension). After laking, the fluid is placed in the incubator at 37° C. in order to evaporate off the ether. Two series of tubes are prepared as before, each tube containing 1 c.c.,—in the one case of the suspension, in the other of the red fluid resulting from laking.

Example.—Two series of tubes, each containing 1 c.c. of suspension of red corpuscles in 0·85 per cent. sodium chloride solution, are prepared. In one series laking is produced by ether and the ether is evaporated off; in the other series the corpuscles are left untreated. 6 doses of immune-body are then added to each of the tubes and increasing amounts of complement,—namely, 0·02, 0·05, 0·1, 0·15, 0·2, 0·25 c.c. of complement.

The result is the same as in the case of laking with water, the tube with 0·15 c.c. of complement giving a small trace of complement over in both series, i.e. the receptors of the laked corpuscles take up as much complement through the medium of immune-body as the receptors of the untreated corpuscles.

Accordingly neither water nor ether in causing laking of the corpuscles produces any change in the affinity of the receptors for immune-body and, through this intermediary, for complement.

Effects of Heat on the Receptors

We have also carried out in a similar manner experiments on the effects of exposing the corpuscles to various temperatures, and may give briefly the results of these. The corpuscles were laked with water, and sodium chloride was added to make up to 0·85 per cent. The red fluid was exposed to various temperatures, the same amount of corpuscles of course being used in each case. *Examples :—*

1. Six hæmolytic doses of immune-body added to each tube. Hæmolytic dose of complement = 0·015 c.c.

1 c.c. of unheated suspension . . .	takes up	0·12 c.c. of complement.
„ of suspension heated for ten minutes at 65° C . . .	„	0·09 c.c. „
„ of suspension heated for ten minutes at 75° C . . .	„	0·07 c.c. „
„ of suspension heated for ten minutes at 85° C . . .	„	0·05 c.c. „

2. Six hæmolytic doses of immune-body, &c., as in 1.

1 c.c. of suspension heated for sixty minutes at 85° C. . . .	takes up	0·042 c.c. of complement.
„ of suspension heated for ten minutes at 100° C. . . .	„	0·045 c.c. „

3. Six hæmolytic doses of immune-body. Hæmolytic dose of complement = 0·02 c.c.

1 c.c. of unheated suspension . . .	takes up	0·17 c.c. of complement.
„ of suspension heated for ninety minutes at 100° C. . . .	„	0·045 c.c. „

Controls were made without any immune-body ; in these there was no appreciable absorption of complement.

These examples show clearly that the receptors vary in their resistance to heat. While on the one hand it appears that a small proportion of the receptors are destroyed by exposure for ten minutes at 65° C., on the other hand a fairly large proportion show a high degree of resistance, about a quarter of the original number withstanding a temperature of 100° C. for over an hour. This latter proportion is probably rather under the real amount, as when the higher temperatures are used brownish flocculi are formed, and therefore both immune-body and complement will have difficulty in penetrating to the centre of these and thus saturating the receptors.

On the Relations of the Receptors to Stromata

1. *Experiments by Centrifugalization.*

Having now a satisfactory test for the presence of receptors, we can determine, after the corpuscles have been lysed, whether on centrifugalization the receptors are to be found in the deposit or are left in the supernatant fluid. We have carried out such experiments both when the corpuscles are laked with water and when lysis is produced by a minimum dose of hæmolytic serum.

(*a*) *Laking with water.*—The corpuscles are laked according to the description given above, sodium chloride being afterwards added so as to make up to the original concentration of 0·85 per cent. The red fluid is then centrifugalized for varying periods, the centrifuge running about 3000 revolutions per minute. The fluid in the tubes is then divided into two equal parts,—the upper clear fluid, and the lower portion containing the deposit. The latter is then thoroughly shaken so as to distribute the stromata equally, and the two fluids are distributed in two series of tubes, each containing 1 c.c. A number (seven to ten) of doses of immune-body are added to each tube, and then to the tubes in series increasing amounts of complement.

The amount of complement taken up is reckoned from the first tube in which complement can be obtained after incubation for two hours at 37° C.

Example.—Centrifugalization of the laked corpuscles is carried on for an hour. To each tube seven doses of immune-body are added. The result is—

(*a*) Upper part of fluid. Amount of complement taken up = 0·09 c.c.
(*b*) Lower part of fluid with stromata „ „ = 0·24 „
M.H.D. of complement . . . . . = 0·016 „

In another case centrifugalization was performed for two hours. Ten doses of immune-body were added to each tube (in this case only 0·5 c.c. of fluid to be tested was placed in each tube).

Upper part of fluid. Amount of complement taken up = 0·007 c.c.
Lower part, with sediment „ „ = 0·29 „
M.H.D. of complement . . . . . = 0·03 „

From such and other experiments we have arrived at the conclusion that it is possible to make a large proportion of the receptors pass to the bottom of the tube, but that it is not possible to free the upper part of the fluid entirely from receptors.

On examining microscopically the upper part of the fluid obtained by centrifugalization, we have generally found a few stromata or shadows, though these have seemed insufficient to explain the amount of complement taken up; in one case we could not detect any. (They are distinguished more easily when some concentrated salt solution is added to the fluid.) We have tested the effect of centrifugalization with a greater strength of salt solution, namely, 1·7 per cent.,—double the usual strength. In such a fluid the stromata become contracted and are deposited more rapidly. Before the power of taking up complement is estimated, the percentage of salt is reduced to 0·85 by adding an equal volume of sterile water. The following may be taken as an example :—

In (*a*) centrifugalization was performed in 0·85 per cent. sodium chloride, in (*b*) in 1·7 per cent. After centrifugalization equal amounts

of fluid were used in the two cases; in (*a*) dilution was made with 0·85 per cent. sodium chloride, in (*b*) with water. M.H.D. of complement = 0·015 c.c.

(*a*) 1 c.c. of upper part of fluid saturated with immune-body took up . . 0·06 c.c. of complement.

(*b*) 1 c.c. of upper part of fluid saturated with immune-body took up . . 0·035 c.c. of complement.

On adding some concentrated salt solution to the fluid in (*a*), some stromata were seen on microscopic examination, but none could be seen in the fluid (*b*).

Such an experiment therefore shows that the freer the fluid is from stromata the fewer are the receptors. *But we have never been able by centrifugalization to obtain a fluid in which no receptors could be demonstrated, even although no stromata could be seen in it on microscopic examination.*

(*b*) *Lysis with immune-serum.*—In this case we add several hæmolytic doses of immune-body, allow time for combination, and then produce lysis with the minimum dose of complement. As has been shown above (p. 16), when this procedure is carried out, the receptors remain in union with immune-body, and thus lead to the taking up of additional complement. We can thus test whether such receptors are deposited in the process of centrifugalization.

Example.—To 10 c.c. of suspension of red corpuscles are added eight times the hæmolytic dose of immune-body. Time is allowed for combination, and then complete lysis is produced by the minimum dose of complement. The fluid is then centrifugalized for two hours, and it is then distributed as 'upper' and 'lower' portions in tubes, 0·5 c.c. being placed in each. The power of taking up complement is then tested as before, with the result:—

(*a*) Upper portion of fluid. Amount of complement taken up . . . . . = 0·05 c.c.

(*b*) Lower portion of fluid with deposit. Amount of complement taken up . . . . . = 0·19 ,,

Other experiments have given similar results, the general conclusion being the same as that in the case of corpuscles laked with water.

2. *Filtration Experiments.*

Attempts to produce *complete* sedimentation of the receptors having thus failed, it occurred to us that it would be of interest to test the effect of passing the fluid containing the laked corpuscles through a porcelain filter. The question simply is—Are the receptors retained in the filter, or do they pass through ? The methods of laking with water and of lysing with hæmolytic serum are exactly the same as those detailed above, so they need not be repeated.

(*a*) *Laking with water.*—The red fluid, made up to a concentration of 0·85 per cent. sodium chloride, is slowly passed through a sterile filter ; 1 c.c. of the filtrate is placed in each of a series of tubes, and as a control we have a series containing simply 1 c.c. of 0·85 per cent. salt solution in each. To each tube in the two series is added several doses of immune-body, and then, as before, increasing amounts of complement, the amount in the first tube being less than a hæmolytic dose. After two hours' incubation, the presence of complement is tested for in the usual way.

The results have varied somewhat. In some cases there was no evidence that any of the receptors had passed through ; that is, the filtrate had no power of taking up complement when immune-body was added to it. In other cases, receptors could quite easily be demonstrated in the filtrate, though never in so great quantity as in the clear fluid obtained by centrifugalization, even with 1·7 per cent. salt solution. We may say that it is exceptional for 1 c.c. of the filtrate to take up a hæmolytic dose of complement, excess of immune-body of course being present. We have endeavoured to ascertain the reason of the variations observed. As one result, we have found that when a quantity of the fluid containing the laked corpuscles is passed through the filter, the portion which passes through first is almost free from receptors, while that passing through

later contains a perceptible amount. We have employed a small Berkefeld filter and a Chamberland, and in both cases the results are of the same nature, though, as one would expect, the receptors are more efficiently retained by the Chamberland filter. The following may be quoted as a characteristic example:—

The corpuscles of 200 c.c. of 5 per cent. of suspension are lysed with water, and the fluid is made up to the original volume and a percentage of 0·85 sodium chloride.

Filtration is then performed by a Chamberland filter, and portions of the first 30 c.c. and the second 20 c.c. respectively are tested for the presence of receptors. The fluid is arranged in a series of tubes, 1 c.c. in each, and five doses of immune-body are added. Complement is then added to the tubes in increasing amounts, and the tubes are allowed to remain in the incubator for two hours. To each tube 1 c.c. of suspension of corpuscles treated with immune-body is added, and the amount of lysis is observed. At the same time varying amounts of complement alone are tested with treated corpuscles in the usual way.

It is found that in the case of the first part of the fluid, 1 c.c. treated with immune-body takes up 0·002 c.c. of complement, while in the case of the second portion the amount is 0·01 c.c. As the full amount of complement taken up by all the receptors saturated with immune-body would have been about 0·2 c.c., this means that in the first portion about 1 per cent. of the receptors passed the filter, and in the second about 5 per cent. We are thus probably safe in concluding that the first few cubic centimetres would be practically free of receptors.

It occurred to us that the length of time during which the fluid was allowed to stand after the corpuscles were laked, might have some influence on the number of receptors which would pass the filter. We have tested this at varying periods, the corpuscles being laked, for example, during a quarter of an hour, twelve hours, and twenty-four hours, and then filtered. We used the same filter, and found that the amount of receptors which passed through depended on the order in which the three fluids were filtered; that is,

when the twenty-four hours' fluid was filtered first the corresponding filtrate contained the fewest receptors of the three, and the corresponding condition obtained when the quarter of an hour fluid was filtered first. We therefore found no evidence that in course of time a greater number of receptors passed into the surrounding fluid when the corpuscles were laked with water.

The explanation of the results stated cannot be considered as quite clear. Of course, as filtration goes on the pores of the filter gradually become choked with stromata and a higher pressure becomes necessary; in this way more of the receptors might be forced through. (On the other hand, it might *a priori* have been considered likely that more receptors would have passed through when the pores of the filter were freer.) It is, however, also possible that the receptors become deposited in some mechanical way on the filter, and we are at present not justified in concluding that the size of particles alone is the determining factor. Experiments which we have made on the filtration of fresh serum show that the amount of complement which passes through varies greatly: in some cases the complement falls only slightly in value, in other cases the greater part is kept back. Immune-body, on the other hand, appears to pass through practically unchanged. Further results obtained in a subsequent research are given above (p. 90). It may be noted that Graham-Smith[1] found on filtering serum through a Berkefeld filter that the amount of precipitable substance in the filtrate first passed was diminished; but that it gradually rose to the original amount after a certain quantity had been passed. In the case of a Chamberland filter the amount of precipitable substance diminished rapidly and fairly uniformly as the filter became choked.

[1] Graham-Smith, *Journ. of Hygiene*, 1903, vol. iii, p. 357.

The conclusions which can be drawn from these observations are, that a fluid containing much fewer receptors can be obtained by filtration than is possible by means of centrifugalization—the filtrate in the earlier stages of filtration being practically free from receptors. On the other hand, it is shown that an appreciable amount of receptors can pass the filter when the process of filtration has continued some time, this amount being greater in the case of the Berkefeld than of the Chamberland filter.

(*b*) *Laking with hæmolytic serum.*—We add multiple doses of immune-body, allow time for combination, then wash and centrifugalize the corpuscles to remove the small amount of uncombined immune-body, and lyse with a minimum dose of complement. The resulting fluid, which takes up further complement according to the amount of immune-body present, is then filtered as before.[1]

The result obtained both with a Berkefeld and a Chamberland filter is that no receptors are present in the filtrate; that is, to put the matter in another form, the filtrate has no power of taking up complement when immune-body is added to it. The problem as to the condition in which the retained receptors are, is the same as in the previous case. The difference between the two methods of lysis would appear to be that the more disruptive action of water has the effect of enabling a small proportion of receptors to pass the filter, especially when filtration is continued for some time.

Another application of the method of filtration may be noted. It has been stated above (p. 16), that when multiple doses of immune-body are united to red corpuscles, the subsequent lysis by a dose of complement does not set free the surplus molecules of immune-body; in other words the combination of immune-body with receptors is not

[1] The pores of the filters become choked more quickly than when the corpuscles are laked with water. The later portions of the filtrate are, however, found to be free from receptors.

affected by lysis. Accordingly, in such a case, if the receptors are retained by the filter the molecules of immune-body which readily pass through in the free condition should be retained also. This is found to be the case. The suspension of red corpuscles is treated with seven hæmolytic doses of immune-body, and the corpuscles are washed and centrifugalized repeatedly to remove the uncombined immune-body ; they are then lysed with a minimum dose of complement. Part of the fluid is filtered, and we then test how much immune-body can be obtained from the unfiltered and filtered fluid respectively by placing red corpuscles in it for an hour at 37° and then adding complement. In the case of the unfiltered fluid a full dose of immune-body can be obtained ; this is obtained by dissociation from the combination receptor + immune-body ; from the filtrate only a tenth of a dose is obtainable. But why should any immune-body be obtained, seeing that the receptors do not pass through the filter, and the immune-body is combined with them ? The answer is that a certain amount of immune-body dissociates from the red corpuscles at 37° C. when they are washed and fresh salt solution is added, and this small amount of free immune-body passes through the filter ; in other words, the combination of immune-body with red corpuscles is not in equilibrium when the fluid is free from immune-body, and a small fraction of the latter dissociates. This can easily be proved by taking red corpuscles combined with several, say eight doses of immune-body, and washing them repeatedly in salt solution to remove the uncombined immune-body. The corpuscles are then divided into two equal portions and fresh salt solution is added. One portion (*a*) is put in the incubator at 37° C., the other (*b*) is kept at 0° C. After an hour the two portions are centrifugalized and the clear fluid from each is separated. It is found that a small fraction of immune-body is present in the fluid obtained from (*a*), this having dissociated at

the higher temperature, while in the fluid from (*b*) no perceptible amount of immune-body is present. This is an additional illustration of the reversibility of the combination, receptor + immune-body.

General Summary

1. By the method employed, which affords a comparatively delicate test for the presence of receptors, it has been shown that the hæmolytic receptors of the red corpuscles are comparatively stable molecules. So far as is shown by their combining affinities, they are not destroyed when the corpuscles are laked by water or by ether ; the fluid resulting from laking having the power of taking up as much complement through the medium of the immune-body as the original suspension had. It is almost unnecessary to point out that this result brings into special prominence the fact that in the case of such hæmolytic sera we have a chemical interaction between three sets of molecules—receptor, immune-body, and complement—and that this interaction can be demonstrated as readily when the corpuscles have been disintegrated by certain laking agents as when they are in the intact condition. The receptors also show considerable stability when exposed to higher temperatures ; while a small proportion are destroyed at 65° C., a considerable number resist exposure to 100° C. for an hour.

2. When corpuscles are lysed with water or a single dose of hæmolytic serum, it can be shown by centrifugalization that the greater number of receptors remain attached to the stromata and become sedimented with them ; the upper clear fluid also, however, contains a considerable number, even although no stromata can be seen on microscopic examination.

3. When red corpuscles are lysed with a single dose of

hæmolytic serum, and the resulting red fluid is passed through a porcelain filter, the filtrate is found to be free from receptors. (The physical means by which they are retained is an open question : it does not follow that the receptors are attached to particles which are too large to pass through.) When the corpuscles are laked with water and the red fluid is filtered, a small fraction of receptors pass through, especially after filtration has been continued for some time. The difference in these two results is probably due to the more disruptive action of the water on the substance of the corpuscles.

PART II

THE PROPERTIES OF AN ANTI-SERUM TO A SERUM; DEVIATION OF COMPLEMENT AND ITS RELATIONS TO THE PRECIPITIN TEST

Recent researches on the subject of immunity, and especially those dealing with hæmolytic sera, have thrown a flood of light on the complicated constitution of serum and other fluids and have given us the means of attacking problems which were, and still are, quite inaccessible to ordinary chemical methods. As has been shown above, when bacteria or the cells of another species of animal are injected into an animal, there are developed immune-bodies which in association with complement or alexine produce the bactericidal or hæmolytic effect. From the point of view of chemical combination the all-important fact is that certain molecules or receptors in bacteria, &c., give rise to anti-substances which lead to the fixation or absorption of complement. It has also been shown that in a normal serum in addition to complement there are also present the homologues both of receptors and of immune-bodies. Accordingly when a serum of one animal is injected into another of different species there is theoretically the possibility of the development of three different kinds of anti-substances. Anti-complements have received most attention hitherto, as their action was the first to be recognized, but recently their existence has been called in question, as will presently be explained. The subject of anti-immune-bodies, pro-

duced by the injection of a normal serum, has been treated of above, and their mode of action has been detailed. In the present section we propose to deal with the anti-substances developed by the receptors of a normal serum, these two bodies in conjunction leading to the absorption or fixation of complement. We shall in the first place refer to the work which has recently been done on this subject.

In 1902 Gengou[1] showed that when an anti-serum was developed by the injection of various albuminoid substances into an animal, the mixture of the substance and the anti-substance might not only give rise to a precipitate, but might also have the power of absorbing complement or alexine. This phenomenon he regarded as analogous to what was known to obtain with regard to hæmolytic sera and bacteriolytic sera, and he spoke of the anti-substances developed in the treated animals as 'sensibilisatrices (immune-bodies) contres les substances albuminoids'. In Ehrlich's terminology we may express this by saying that the receptors in the albuminoid molecules give rise to immune-bodies or amboceptors and that the combination of the two takes up complement. Gengou obtained this result with milk, egg-white, fibrinogen, and the serum of another species of animal than that injected. Special attention has recently been drawn to the subject by a paper by Moreschi[2] on the nature of anti-complements. An 'anti-complement' obtained by injecting a normal serum acts chiefly, as is well known, on the serum ('complement') injected; but Moreschi has shown that if a minute quantity of the homologous serum is added to the anti-serum various complements may be taken up—that is, antagonized—or, in other words, the anti-serum behaves as an anti-complement to various complements. He points out that an extremely minute quan-

[1] Gengou, *Annales de l'Inst. Pasteur*, 1902, p. 734.
[2] Moreschi, *Berlin. klin. Wochenschr.*, 1905, p. 1,181.

tity (0·000,01 c.c.) of the original normal serum added to the anti-serum may result in the taking up or deviation of various complements. From his experiments he has found reason to doubt whether there is any real anti-complement in the strict sense, i.e. a substance which unites directly with a complement and thus prevents its action, the apparently anti-complement action being a deviation phenomenon. Neisser and Sachs[1] have applied Moreschi's results to the differentiation of the bloods of different species of animals, and have shown that the deviation of complement is a much more delicate test than the precipitation test; that is, a much smaller amount of the serum (precipitinogen) when added to the anti-serum (precipitin) will produce a deviation or fixation of complement than that necessary to cause a visible precipitate. They consider that the phenomenon is analogous to the fixation of complement by a cell-receptor when combined with its corresponding amboceptor. Gay[2] regards the precipitate as the all-important factor in the fixation of complement, and finds that when a precipitate forms the separated fluid is without effect on complement, whilst the precipitate fixes or combines with a considerable quantity. He extends his observations with the purpose of showing that the phenomenon of deviation in hæmolytic and bactericidal experiments may depend upon fixation by precipitate. Pfeiffer and Moreschi[3] have found that the precipitate, by fixing complement, has an anti-bacteriolytic action in the animal body. In a second communication on anti-complements Moreschi[4] considers the quantitative relationships between the serum and anti-serum (precipitinogen

[1] Neisser and Sachs, *Berlin. klin. Wochenschr.*, 1905, No. 44, and 1906, No. 3.

[2] Gay, *Centralbl. f. Bakteriol.*, Abtheil I., Originale, 1905, vol. xxxix, p. 603; *Annales de l'Inst. Pasteur*, 1905, p. 593.

[3] Pfeiffer and Moreschi, *Berlin. klin. Wochenschr.*, 1906, p. 33.

[4] Moreschi, ibid., 1906, p. 76.

and precipitin) in relation to the absorption of complement, and comes to the conclusion that these unite in variable proportions. He also emphasizes the parallelism between the amount of precipitate formed and the amount of complement (alexine) absorbed. Friedberger[1] gives an account of his observations on this subject, one of the most important of which is that, while most of his anti-sera have shown deviating powers similar to those recorded by others and observed by ourselves, he has obtained an anti-human serum which gives a deviation in the extraordinarily minute amount of 0·000,000,001 c.c. Even 0·000,01 c.c. of human sweat produced a recognizable fixation of complement with this anti-serum. This is further referred to below. The subject is critically reviewed by Liefmann[2] especially in relation to precipitum-formation on the one hand and amboceptor-action on the other. He considers that a satisfactory explanation of the fixation of complement is not yet possible.

It thus appears that a number of questions, both of practical and of theoretical importance, are opened up by these investigations and we shall deal with some of them in this section.

Methods. The method which we used for preparing the anti-serum is that usually followed in precipitin work, viz. the intra-peritoneal injection of a particular serum in varying doses at suitable intervals of time. The anti-serum before being used is of course heated at 55° C. to destroy its complement. We have used three anti-sera obtained from the rabbit, which act on the serum of man, the ox, and the guinea-pig, respectively, and one from the guinea-pig acting on rabbit's serum. We may conveniently represent the first of these anti-sera as *anti-serum rabbit v. man*, and so with

[1] Friedberger, *Deutsche med. Wochenschr.*, 1906, p. 578.
[2] Liefmann, *Berlin. klin. Wochenschr.*, 1906, p. 448.

the others. The method of testing the deviation of complement is carried out in two stages. (1) To each of a series of test-tubes a given amount of the anti-serum, usually 0·05 c.c., is added along with a given amount of the homologous serum, and the volume is made up with 0·8 per cent solution of sodium chloride to 1 c.c. To the several tubes varying amounts of complement (normal serum of rabbit or guinea-pig) are added, the smallest amount being about the minimum hæmolytic dose for the amount of red corpuscles to be afterwards used in the test. It will be seen that in this stage there is always a mixture of three substances, the serum, the anti-serum, and the complement whose absorption is to be observed. The tubes are placed in the incubator at 37° C. for one and a half to two hours to allow time for combination. (2) At the end of that time 1 c.c. of a 5 per cent. suspension of red corpuscles treated with the corresponding immune-body is added to each tube and the tubes are again placed in the incubator for one and a half hours. We can thus observe the tube in which there is the first trace of lysis, and the tube in which lysis is first complete. It is convenient to prepare several series of tubes with different amounts of the homologous serum, 0·01, 0·001, 0·000,1 c.c., &c., in each series. In each experiment a control series with anti-serum along with different amounts of complement alone is prepared, and the difference between the lysis in this and the other series is interpreted as being due to the presence of the homologous serum. We also have in each series a tube containing corresponding amounts of serum and anti-serum alone, to show precipitation. It is evident that the conditions of experiment may be varied by making the amount of the homologous serum (precipitinogen) fixed, and varying the amount of anti-serum.

It is thus seen that we have in such experiments two substances and their anti-substances, viz. serum + anti-serum and red corpuscles + immune-body: opportunity is given to

the complement to unite with the first combination and then the presence of free complement is tested for by its hæmolytic effect.

1. Phenomena of Deviation of Complement

The following may be taken as a typical example of a deviation experiment :—

Anti-serum, rabbit *v.* ox, 0·05 c.c. to each tube.
Serum of ox 55° C.,[1] 0·01 — 0·000,001 c.c.
Deviation of guinea-pig's complement.
Test for complement = 1 c.c. suspension of ox corpuscles + immune-body. Minimum hæmolytic dose of complement = 0·01 c.c.

Table 1

Anti-serum c.c.	*Ox-serum* 55° C. c.c.	*Amounts of complement* 0·01	0·02	0·03	0·04	0·05	0·06 c.c.	Amount of lysis in added corpuscles
0·05	0	$\frac{7}{10}$	complete	complete	complete	complete	complete	
0·05	0·000,001	$\frac{2}{5}$	almost complete	complete	complete	complete	complete	
0·05	0·000,01	trace	$\frac{3}{4}$	just complete	complete	complete	complete	
0·05	0·000,1	0	$\frac{1}{2}$	$\frac{3}{4}$	nearly complete	complete	complete	
0·05	0·001	0	0	0	0	slight trace	trace	
0·05	0·01	0	0	0	0	0	0	

The amount of lysis indicated in this and other tables is of course due to the amount of complement left free after contact with the serum and its anti-serum for one and a half hours at 37° C. The results of this experiment are graphically represented in the accompanying figure (Fig. 1, p. 139).

It will be seen from the amounts of the resulting lysis given in the table that without any of the ox's serum 0·01 c.c. of complement gives $\frac{7}{10}$ lysis of the added corpuscles, whilst the addition of 0·000,001 c.c. of ox serum reduces the lysis to $\frac{4}{10}$; that is, this amount of serum in combination with the anti-serum has deviated about a third of a dose of complement.

[1] In each case the serum used is heated to 55° C. for an hour at least, to destroy the complement naturally present.

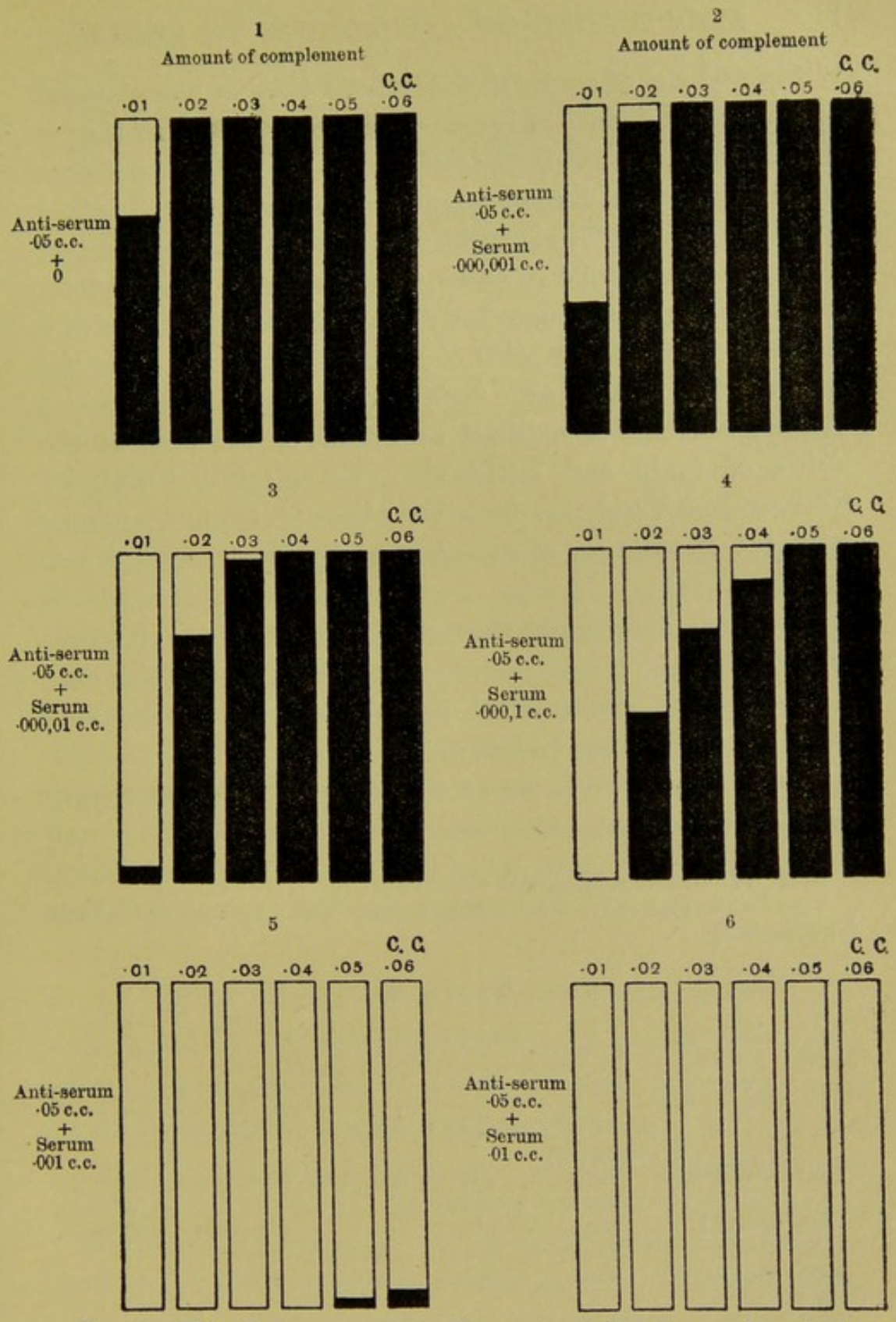

Fig. 1. This figure represents diagrammatically the results of the experiment given in Table 1. The columns indicate test-tubes, and the height of the black portion shows the amount of lysis of the test corpuscles in each. The amounts of serum, anti-serum, and complement are also given.

With 0·001 c.c. of serum six hæmolytic doses of complement produce only a trace of lysis. It is also to be noted that if we take the first tube in the series where any lysis is present, much more than a dose of complement must be added before lysis is complete : for example, with 0·000,1 c.c. of serum two doses must be added in order that lysis may be complete. If we suppose that a new body is formed by the union of a molecule in the serum with one in the anti-serum, then the combination of this new body with complement presents phenomena well recognized to obtain in the case of the union of toxin and anti-toxin—the so-called ' Ehrlich's phenomenon ', and also described above (p. 32) in connexion with the absorption of complement by sensitized red corpuscles.

The table also shows that increasing amounts of serum lead to the taking up of more complement, though this does not occur in arithmetical proportion. This point will, however, be referred to below.

The following table shows the result of a similar experiment with anti-human serum.

Anti-serum rabbit *v.* man, 0·05 c.c. to each tube.
Test for complement = 0·5 c.c. suspension of ox's corpuscles treated with immune-body.

TABLE 2

Anti-serum c.c.	*Human serum* c.c.	*Rabbit's complement* 0·05	0·1	0·2	0·3 c.c.	
0·05	0	practically complete	complete	complete	complete	Lysis in added corpuscles
0·05	0·000,01	very slight	just complete	complete	complete	
0·05	0·000,1	none	none	⅓	complete	
0·05	0·001	none	none	none	none	
0·05	0·01	none	none	none	none	

From the above it will be seen that 0·000,01 c.c. human serum produces distinct deviation. Precipitin tests were carried out at the same time with the result that 0·000,1 c.c.

gave only a very slight, in fact rather doubtful precipitate, whilst 0·000,01 c.c. gave no trace of precipitate.

Table 3 shows the action of our third serum.

Anti-serum, rabbit *v.* guinea-pig.
Test for complement = 0·5 c.c. suspension of ox's corpuscles treated with immune-body.

TABLE 3

Anti-serum c.c.	*Guinea-pig's serum* c.c.	*Rabbit's complement* 0·05	0·075	0·1	0·15 c.c.	
0·04	0	0	0	almost complete	complete	Lysis in added corpuscles
0·04	0·000,01	0	0	0	0	
0·04	0·000,1	0	0	0	0	

In this case the anti-serum alone has a slight though distinct effect on the hæmolytic action of rabbit's complement; this is probably due to the fact that rabbit's and guinea-pig's sera contain a few common receptors. The addition of even 0·000,01 c.c. of guinea-pig's serum 55°, however, gives a marked deviation phenomenon. This experiment is of special interest as the anti-serum used gave practically *no precipitate* when added to the homologous serum; even 0·01 c.c. of the latter produced merely a faint cloudiness but no real precipitate.

2. On the Deviation of different Complements

It can be readily tested by the above methods whether or not any given complement is taken up by a particular combination of serum + anti-serum. Moreschi found that a number of different complements may be deviated by the same combination, or, as he expresses it, an anti-serum becomes anti-complement to the complements of different animals on the addition of a small quantity of the homologous serum. Our observations are confirmatory of this, but they show also that some complements may not be deviated. The red corpuscles used in testing for comple-

ment may be treated with an immune-body artificially developed for these corpuscles, or we may depend in some instances on the natural lysis which may be produced by a normal serum. (In this latter case there is of course in the serum a natural immune-body or Zwischenkörper which acts along with the complement.) The following may be cited as examples. The anti-serum rabbit *v.* ox (along with the homologous serum) deviates (*a*) guinea-pig's complement as tested either with ox's or rabbit's corpuscles treated with the corresponding immune-body, and (*b*) rabbit's complement when tested in the same way ; it also deviates (*c*) dog's complement as tested by the natural lysis of dog's serum on rabbit's corpuscles. The anti-serum rabbit *v.* man deviates both (*a*) rabbit's and (*b*) guinea-pig's complement when tested with ox's corpuscles treated with immune-body, and also (*c*) cat's complement when tested by the natural lysis of guinea-pig's corpuscles by cat's serum. If we regard the specific substance in the anti-serum as the homologue of an immune-body, these results show that many complements are taken up through the medium of the same immune-body. Analogous results are obtained in the case of hæmolytic immune-bodies.

We have, however, met with the following two exceptions, though we have made no very extended series of observations, and probably many others will be found to obtain. The anti-serum rabbit *v.* ox (along with the homologous serum) does not deviate ox's complement in the natural lysis of rabbit's corpuscles by ox's serum. Again, the anti-serum rabbit *v.* guinea-pig does not deviate rabbit's complement when guinea-pig's corpuscles treated with immune-body from the rabbit are used as the test[1]. *It is thus*

[1] In this case there is a striking analogy to what has been described above (p. 77) in the case of hæmolytic immune-bodies, viz. that increased amounts of immune-body for guinea-pig's corpuscles did not take up (or deviate) increased amounts of rabbit's complement when guinea-pig's corpuscles + immune-body were used as the test, whereas

shown that many, but not all, complements are taken up by the combination of a serum with its anti-serum.

Another point worthy of note is that we have observed an apparent variation in the firmness of union of the complement deviated. This is indicated by the manner in which the lysis progresses when the test corpuscles are added; in some cases the lysis comes to an end after an hour or an hour and a half at 37° C., in others it continues to increase, as if the complement were being separated from the combination of serum + anti-serum molecules. For example, with the anti-serum rabbit *v.* ox along with the homologous serum, the combination of guinea-pig's complement appeared to be firmer than that of rabbit's complement, whereas with the anti-serum rabbit *v.* man the converse was the case. The results, in short, point to the possibility in some cases of complement becoming dissociated from the combination serum + anti-serum, or, in other words, the deviation of complement may exhibit varying degrees of permanency.

3. Deviating Power as compared with Hæmolytic Action

As already stated, the union of the molecules in the anti-serum with those in the homologous serum leads to the taking up of complement. In this we have a close analogy to what occurs in the case of a hæmolytic serum, where by the union of the receptors of the red corpuscles with immune-body, complement enters into combination, this combination, as has been shown, being generally of firm nature. Apparently then, when a serum is injected into an animal, there are formed molecules which are the homologues of immune-bodies. The amount of serum along with its anti-

they did so when ox's corpuscles + immune-body were used. In fact, if we substitute anti-serum to guinea-pig's serum for immune-body to guinea-pig's corpuscles the results coincide in the two cases.

serum necessary to produce deviation is, however, out of all proportion smaller than the amount of red corpuscles (treated with immune-body) necessary to show an appreciable absorption of complement. There are also differences, to be mentioned below, when the combination of the molecules and anti-molecules is used in varying proportions in the two cases. It has been shown by Morgenroth[1] that a hæmolytic serum may be developed by the injection of serum, the latter apparently containing receptors with the same combining-group as the hæmolytic receptors of the red corpuscles. The anti-serum to ox's serum used by us has hæmolytic action, the minimum hæmolytic dose for ox's corpuscles being 0·05 c.c. ; as already stated, it gives deviation of complement with 0·000,001 c.c. of ox's serum. The hæmolytic serum acting on ox's corpuscles has a minimum hæmolytic dose of 0·001, 5 c.c.: it also gives deviation along with ox's serum, but not with a smaller dose than 0·001 c.c. of the latter. The former anti-serum has thus only about a thirtieth of the hæmolytic action of the latter, but has about a thousand times more deviating power when tested with the homologous serum. Furthermore, if the anti-serum to ox's serum be left for a time in contact with a sufficient amount of ox's corpuscles, practically all the hæmolytic immune-body can be removed, but the precipitating and deviating properties remain in the serum. It is thus evident that *the molecules in the serum which, in association with the anti-serum, deviate complement in such experiments, are different from the hæmolytic receptors.*[2]

[1] Morgenroth, *München. med. Wochenschr.*, No. 25, 1902.

[2] Gay has suggested that probably many errors have arisen in hæmolytic experiments through non-recognition of the deviation of complement by a serum+its anti-serum, and has pointed out the difficulty in freeing red corpuscles completely from the serum by washing. Even, however, if a small quantity of serum be left, the amount of anti-serum necessary to produce deviation is relatively great, and as all the hæmolytic sera which I have used have been powerful (0·003 c.c. being generally the hæmolytic dose), I am certain that no error of importance can have arisen

4. Relation of the Deviation of Complement to Precipitation

That the union of the two substances concerned in the fixation of complement is often attended with precipitation has been recognized by various observers. Moreschi in his first paper states that the deviation phenomenon appears only as a sequel to precipitation and stands in closest relation to it; in his second publication, however, he speaks less decidedly on this point, though he says that the amount of complement fixed is always in proportion to the amount of precipitate. Neisser and Sachs in describing the application of the deviation test for differentiating the blood of different species regard the results by the two methods as analogous, but consider that the essential in the phenomenon of deviation is the union of a substance and its anti-substance (amboceptor). Gay speaks of the 'fixation of alexins by specific serum precipitates' and finds, as stated above, that the precipitate separated and washed takes up complement. We shall first state the facts which we have observed, and afterwards consider their significance.

In the first place we may consider the relative delicacy of the two reactions. All observers who have written on the subject state that the deviation test is more delicate than the precipitin test, and our results agree with this. In making the comparison the occurrence of precipitation is observed by using the same amount of anti-serum (generally 0·05 c.c.) in each case along with varying amounts 0·01, 0·001, 0·000,1, 0·000,01 c.c., &c., of the homologous serum, the volume being made up with salt solution to 1 c.c. The tubes are placed in the incubator for one and a half hours

from this cause in the work which I have published. Our views regarding anti-complements, however, require revision, in view of the results established with regard to deviation of complements.—R.M.

as in the deviation experiments, and then in the refrigerator till next morning, when the results are read off. With 0·05 c.c. of our anti-serum rabbit *v.* ox a distinct precipitate is got on the addition of 0·001 c.c. of ox's serum, a very faint precipitate with 0·000,1 c.c., this latter being scarcely reliable for practical purposes. Distinct deviation of rabbit's and guinea-pig's complement is got with 0·000,01 c.c. of ox's serum and appreciable deviation sometimes even with 0·000,001 c.c. With the serum rabbit *v.* man 0·05 c.c. precipitation is distinct with 0·001 c.c. of human serum, almost absent with 0·000,1 c.c. ; deviation of complement is always got with 0·000,01 c.c. We may thus state that in the case of these two sera the deviation test is between ten and a hundred times more delicate than the precipitin test. Neisser and Sachs in the case of an anti-human serum considered that the deviation test was about forty times more delicate, and Friedberger found an even greater difference between the two. The figures which we have stated may be taken as well within the limits, as we have taken the smallest amount of serum which gives a *distinct* deviation. Furthermore, owing to the nature of the reaction the result is much more easily appreciated than in the case of precipitins, especially when there is any natural cloudiness of the serum.

We have also found that the phenomenon of deviation may be well marked in the case of an anti-serum which gives no precipitate. Nuttall states that when the animal used for injection is of closely allied species to that from which the serum is taken a precipitin is not usually developed. We have obtained a result which confirms this in the case of the anti-serum rabbit *v.* guinea-pig. This anti-serum produces no distinct precipitate even when a comparatively large amount—e. g. 0·01 c.c. of the homologous serum is added ; at the most there is only some opalescence, and if the tubes be allowed to stand for twenty-four hours there

is no distinct deposit. It will be seen from Table 3 that with this anti-serum, which produces no precipitate on addition to the homologous serum, the phenomenon of deviation is produced by exceedingly small amounts of the latter, viz. 0·000,01 c.c. This shows that the injection of the guinea-pig's serum into the rabbit gives rise to anti-substances, although the latter cannot be demonstrated by the phenomenon of precipitation. We also found in the course of immunizing a rabbit against human serum that the deviating power appeared before any precipitating action was detectable. We may state, however, that when a precipitate is formed we have always found that it had the property of fixing complement—the test of course being made after the precipitate had been washed and centrifugalized several times. In fact the use of a separated precipitate supplies a very convenient method for freeing a serum of complement. We have, for example, been able by this means to deprive guinea-pig's complement of practically all hæmolytic power for ox's corpuscles treated with immune-body, though the hæmolytic dose of such a complement for 1 c.c. of suspension of red corpuscles may be as low as 0·01 c.c. That the precipitate when formed, or rather molecules in the precipitate, have the power of fixing complement, there is therefore no doubt. Are there any molecules with this property in the separated fluid? To each of a series of tubes 0·025 c.c. anti-serum rabbit *v.* ox and 0·001 c.c. of ox's serum were added, the mixture being made up to 1 c.c. in each tube with salt solution. Another similar series was prepared with 0·01 c.c. ox's serum instead of 0·001 c.c. The tubes were incubated for two hours and then placed in the refrigerator till next morning. The tubes were then centrifugalized and the supernatant fluid was carefully pipetted off from the precipitate in each tube. To the fluid thus obtained different doses of guinea-pig's complement were added and after incubation deviation was

tested for in the usual way. The result in both series was negative, i.e. the molecules which fix complement were practically all in the precipitate. In another experiment with 0·01 c.c. ox's serum and 0·025 c.c. anti-serum, performed in the same way, we found that the supernatant fluid deviated about one-twelfth of a hæmolytic dose of guinea-pig's complement—an exceedingly small amount. So also in the case of anti-human serum we found that the precipitate obtained by mixing 0·1 c.c. of human serum with 1 c.c. of the anti-serum in 5 c.c. of salt solution, possessed exclusively the deviating power, the separated fluid being practically without any effect when tested. These results are in harmony with those obtained by Gay on this point.

We may also add that there is no question of complement being carried down mechanically by the precipitate in process of formation. The precipitate after it has formed may be repeatedly washed and still retains the property of fixing complement.

Observations on the relation between precipitate formed and the deviating power show that the amounts are not strictly proportional. For example, using 0·025c.c. of anti-ox serum along with varying amounts (0·1, 0·01 c.c. &c.) of ox's serum, we found that the maximum deviation of complement was given by 0·001 c.c., whilst distinctly the greatest amount of precipitate was given by 0·01 c.c. Again, on using the same quantity of serum, viz. 0·001 c.c. and varying the amounts of anti-serum, we found that a much greater amount of precipitate was given by 0·3 c.c. of anti-serum than by 0·1 c.c., whilst the amount of complement fixed was practically the same in the two cases. Further details on this point are given below under Section 6. It is also interesting to note that Friedberger and Liefmann have found that it is possible by heating to deprive an anti-serum of its precipitating action while its power of fixing

complement in association with the homologous serum may be retained.

We therefore conclude that (1) when a precipitate forms, the deviating property is contained in it, and may be so exclusively; on the other hand, (2) the deviation phenomenon may occur without precipitation, and (3) the amount of deviation is not always in proportion to the amount of precipitate. The last-mentioned fact would indicate, as Moreschi suggests, that the precipitate has not always the same composition, and possibly the precipitin and the precipitinogen unite in varying proportions.

5. The Deviation of Complement as regards Specificity

The important practical question, with regard to the deviation of complement, is the same as in the case of precipitins, and concerns the possibility of distinguishing different kinds of bloods, or rather sera. We have carried out a number of observations on this subject, though these must be regarded as of a preliminary nature, and a much more extended series will be necessary. Using an anti-human serum, we have tested the sera of various animals with it, and observed whether there was any deviation of rabbit's complement (ox's corpuscles treated with immune-body from the rabbit being used as the indicator). We have obtained purely negative results with the sera of the ox, sheep, pig, dog, cat, mouse, guinea-pig, horse, and pigeon. In each case 0·05 c.c. of the anti-serum was used along with as much as 0·01 c.c. of the serum to be tested: in every case as much complement was found to be free as when the anti-serum was used alone. In the case of the primates, however, distinct deviation of complement was obtained. With the serum of a chimpanzee, for which we are indebted to Prof. Woodhead, the following are

the results obtained, the details being as in former tables :—

Human anti-serum ·05 c.c. to each tube.
The indicator was ·5 c.c. suspension of red corpuscles of ox treated with immune-body from rabbit.

TABLE 4

Anti-serum c.c.	*Chimpanzee serum* c.c.	*Complement of rabbit* 0·05	0·1	0·2 c.c.	
0·05	0	just complete	complete	complete	Lysis in added corpuscles
0·05	0·001	none	half lysis	complete	
0·05	0·000,1	none	complete	complete	
0·05	0·000,01	just complete	complete	complete	

It is thus seen that 0·000,01 c.c. gives no perceptible deviation, whilst 0·000,1 c.c. deviates a hæmolytic dose. In a corresponding test made with human serum, it was found that 0·000,01 c.c. of human serum produced the same deviation as 0·000,1 c.c. of chimpanzee serum. We have also tested the serum of a monkey, viz. *Macacus rhesus*, for several samples of whose blood we are indebted to Mr. Jolly of the Physiological Department of the University of Edinburgh, and were rather surprised to find that it had practically the same deviating power as the chimpanzee serum. Several tests were made, and on every occasion with similar result. Neisser and Sachs also obtained negative results with the bloods of the rat, pig, goat, rabbit, ox, and horse, when these were tested with an anti-human serum. With monkey's serum an interference with hæmolysis was however obtained, the amounts of monkey's serum being rather more than ten times the amount of human serum necessary to obtain the same result : the species of monkey is not stated.

With regard to precipitates, the results of observations made at the same time are shown in the following table :—

Anti-human serum 0·05 c.c. to each tube.

TABLE 5

Amount of serum	*Human serum*	*Chimpanzee serum*	*Macacus serum*
0·000,1 c.c.	? Slight opalescence	0	0
0·001 ,,	Distinct precipitate	Slight but distinct	? Slight opalescence
0·01 ,,	Marked	Marked, less than with human	Distinct though slight

These results are much in conformity with what Nuttall [1] obtained.

Whilst, however, the deviation test places the chimpanzee and the macacus monkey in practically the same relation to man, the precipitation test brings out a difference, the chimpanzee serum giving a more marked reaction with anti-human serum than the macacus serum does. No doubt other analogous results will be found to obtain.

We have also tested the *anti-ox serum* with the sera of some other animals. Using 0·01 c.c. and 0·001 c.c. of the serum to be tested, we obtained no deviation with the serum of the horse, pig, cat, mouse, pigeon and of man. (Larger amounts of serum than those mentioned were not used, as complications may arise from the added serum interfering with lysis.) With the serum of the sheep, however, a deviation was obtained approximating in degree to that given by ox's serum. With 0·05 c.c. anti-ox serum, 0·000,01 c.c. sheep's serum as well as 0·000,01 c.c. ox's serum gave a slight though distinct deviation of guinea-pig's complement, but this was more marked in the case of the ox's serum. A greater difference was, however, brought out when we tested the amount of deviation with a larger amount of the two sera, viz. 0·001 c.c. (the same quantity of anti-serum, 0·05 c.c., being used). In this test we found that six hæmolytic doses of guinea-pig's complement in the case of the ox serum and four hæmolytic doses in the case of the sheep's serum, had to be added before one free dose

[1] Nuttall, *Blood Immunity and Relationship*, Cambridge, 1904, p. 165.

was obtained. With the precipitin test analogous differences were obtained—a slight precipitate was given by 0·000,1 c.c. of both sera, more distinct in the case of the ox's serum; a doubtful trace of precipitate with 0·000,01 c.c. of ox's serum, and no precipitate with 0·000,01 c.c. of sheep's serum. These results also as regards precipitation are in accordance with those obtained by Nuttall. In the case, therefore, of ox's and sheep's serum as tested by anti-ox serum there is a close parallelism between results obtained by the deviation and the precipitation tests.

It may also be mentioned that of a number of sera tested with an anti-goat serum, Moreschi found that the only one, besides the goat's serum, which gave a deviation result was ox's serum.

Although our observations on this subject have been as yet comparatively restricted in scope, they have been sufficient to show a harmony in the results brought out by the two methods. It is quite likely that in a more extended inquiry, one method might, in certain instances, bring out differences with regard to blood relationships which the other fails to do (as for example we found in a comparison of the serum of the chimpanzee and the serum of the macacus monkey).

With regard to the application of the deviation test to forensic purposes, we have practically nothing to add to what has been stated by Neisser and Sachs. It is an important adjuvant to the precipitin method, and will undoubtedly be of great service when there is any cloudiness in the fluid to be tested; the non-occurrence of hæmolysis is a phenomenon so much more easily appreciated than the formation of a slight precipitate. Undoubtedly also the deviation method is considerably more delicate, and Friedberger in the paper above quoted points out that its extreme delicacy, when a very powerful anti-serum is obtained, may be a source of fallacy, as he has obtained reactions with human

sweat. For this reason he advises the use of anti-sera which give deviation with 0·000,01 c.c. of the homologous serum as a minimum ; with a serum of this strength there is no risk of error such as might arise from the material to be tested being impregnated with sweat.

6. On the Quantitative Relations of Serum and Anti-serum to the Deviation of Complement

In the tables given above it appears that the amount of deviation of complement increases, though not in arithmetical ratio, with the amount of serum, when the amount of anti-serum is kept constant. If, however, comparatively large amounts of the homologous serum be used, the amount of complement taken up again becomes diminished as the amount of serum increases. We have carried out a number of observations on this subject, one or two examples of which will exemplify the phenomenon. The results are confirmatory of those recently published by Moreschi, though the details of experiment are somewhat different.

Anti-serum, rabbit *v.* ox, 0·025 c.c. to each tube, with varying amounts of ox's serum 55° C.

Test for complement = 1 c.c. suspension of ox's corpuscles treated with immune-body ; the dose of complement for this is 0·0075 c.c.

Table 6

Anti-serum c.c.	*Ox-serum* 55°C. c.c.	*Guinea-pig's complement* 0·0075	0·015	0·03	0·05	0·07 c.c.	
0·025	0·1	very slight	$\frac{2}{3}$	complete	complete	complete	Lysis in added corpuscles
0·025	0·01	0	$\frac{1}{4}$	$\frac{3}{4}$	nearly complete	complete	
0·025	0·001	0	very slight	$\frac{1}{6}$	$\frac{1}{3}$	$\frac{1}{2}$	
0·025	0·000,1	0	$\frac{1}{4}$	$\frac{1}{2}$	$\frac{3}{4}$	just complete	
0·025	0·000,01	$\frac{1}{2}$	$\frac{2}{3}$	just complete	complete	complete	

It is thus seen that with a given quantity of anti-serum, 0·025 c.c. in this case, there is an optimum amount of serum which gives the maximum deviation, viz. 0·001 c.c., whilst

above as well as below that optimum the amount of deviation diminishes. The maximum precipitate was given by 0·01 c.c. The results of this experiment are graphically represented in Figure 2.

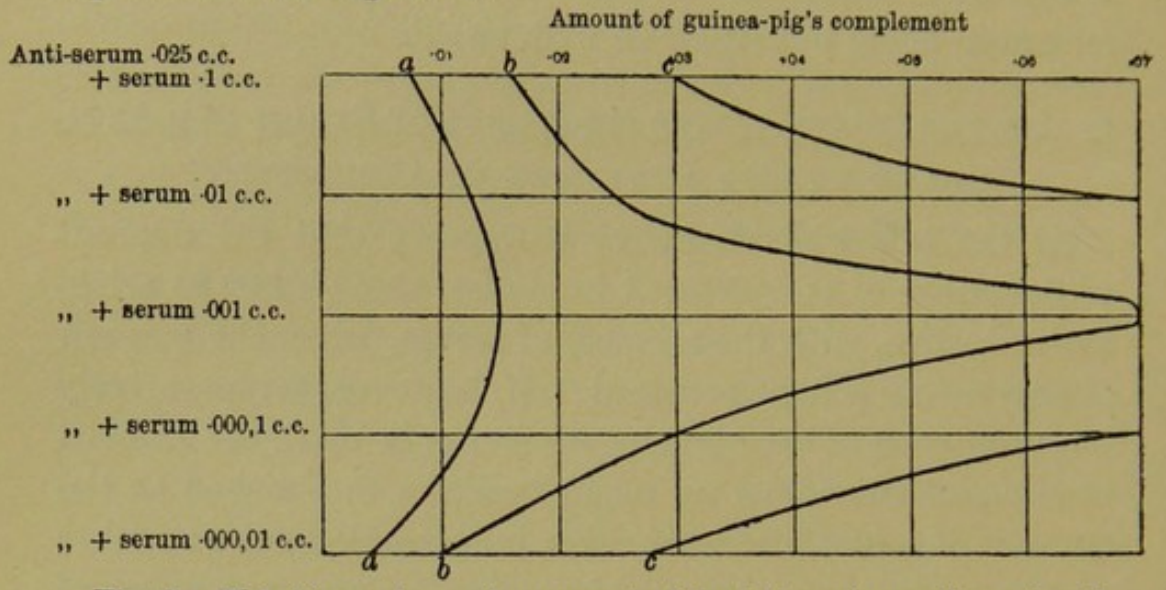

Fig. 2. This figure shows the amount of deviation of complement with 0·025 c.c. of anti-serum and varying amounts of homologous serum. The maximum deviation is given with 0·001 c.c.

curve *a* = initial lysis,
b = half lysis,
c = complete lysis.

A similar result was obtained with anti-human serum, as is shown in the following table.

Anti-serum rabbit *v.* man, ·025 c.c. to each tube. Varying amounts of human serum.

TABLE 7

Anti-serum c.c.	*Human serum* 55° C. c.c.	*Guinea-pig's complement* 0·01	0·02	0·03	0·04	0·05 cc.	
0·025	0·1	—	—	complete	complete	complete	Lysis in added corpuscles
0·025	0·01	—	—	—	complete	complete	
0·025	0·001	—	—	—	—	complete	
0·025	0·000,1	—	—	complete	complete	complete	
0·025	0·000,01	—	complete	complete	complete	complete	

In this experiment there was some diffusion of hæmoglobin owing to the corpuscles having been kept too long; the tubes showing *complete* lysis are accordingly only given. The maximum deviation was thus given by 0·001 c.c. of

serum. On the other hand, 0·01 c.c. gave the maximum precipitate.

We have shown above that complement may be fixed by a serum plus its anti-serum even although there be no precipitate. We have accordingly inquired whether in this instance the principle of optimum proportions holds, and have found that this is the case. The following may be taken in illustration :—

Anti-serum, rabbit *v.* guinea-pig, 0·025 c.c. to each tube. Varying amounts of guinea-pig's serum.

Test for complement = 0·5 c.c. suspension of ox's corpuscles treated with immune-body.

TABLE 8

Anti-serum c.c.	*Guinea-pig's serum* 55°C c.c.	*Rabbit's complement* 0·05	0·1	0·2	0·3	0·4 c.c.	
0·025	0·1	⅓	complete	complete	complete	complete	Lysis in added corpuscles
0·025	0·01	0	0	complete	complete	complete	
0·025	0·001	0	0	½	just complete	complete	
0·025	0·000,1	0	just complete	complete	complete	complete	
0·025	0·000,01	almost complete	complete	complete	complete	complete	

In this case also it will be seen that the maximum deviation occurs with 0·001 c.c. serum, whilst above, as well as below, this amount the deviation of complement diminishes.

It has been recognized by various observers in testing a precipitin (anti-serum) with a given amount of the homologous serum, that the precipitate may become less when the amount of serum is increased beyond a certain point, and after a precipitate has formed this may be dissolved on adding homologous serum. For example, with 0·001 c.c. ox's serum and 0·05 c.c. anti-serum a bulky precipitate is obtained, but this is dissolved in great part on the addition of 0·1 c.c. ox's serum. In this respect also there is an analogy between the phenomena of precipitation and of deviation of complement. We cannot, however, agree with Moreschi

when he says that the amount of complement fixed always depends upon the amount of precipitate, as the results above given show that the maxima of the two reactions may not correspond.

We may vary the conditions of experiment by keeping the amount of serum fixed and varying the amount of anti-serum. In this case Moreschi also 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 anti-serum resulted in diminution in the amount of complement taken up. We have made a large number of observations on this point, but with varying results. In one or two instances we found a slight diminution in the amount of complement deviated, as the anti-serum was increased, but this was never very marked; whilst in the majority of cases we found no such diminution, even when as much as 0·3 c.c. anti-serum was added. At present we cannot give any explanation of this discrepancy. Certainly the phenomena of optimum deviation do not occur in the same striking manner as when the amount of anti-serum is kept fixed and the amount of homologous serum is varied. As stated above, however, we found when we continued to increase the amount of anti-serum (the homologous serum being kept fixed) that the amount of precipitate formed might continue to increase, whilst the deviation of complement did not do so.

General Considerations

The deviation of complement by a serum plus its anti-serum (presumably by a new compound formed) is one of the most striking of serum reactions, and opens up many questions of high theoretical importance. The relation of the phenomenon to precipitation has already been discussed at some length, and whilst there is a certain parallelism between them, we cannot say that it is the precipitate which

fixes complement. The fact that deviation may occur where there is no precipitation would indicate that when a serum is injected the all-important result is the development of anti-substances, and these in combination with certain substances in the serum have the property of fixing complement. The combination of substance + anti-substance may be, and usually is, attended by precipitation. Further, the results with regard to the relation of the amount of complement fixed to the amount of precipitate, suggest that the latter may vary in composition. An analogy can be drawn between the anti-substances in question and the hæmolytic and bacteriolytic immune-bodies. A striking difference, however, is presented by the fact that increase in the amount of serum (receptors) beyond a certain point leads to a diminution in the amount of complement taken up—a phenomenon which so far as we know has not been observed in the case of other anti-sera. The extraordinarily small amount of the homologous serum which is sufficient to produce a recognizable deviation of complement also appears unique in serum reactions.

It has been recognized for a considerable time that when the serum of an animal is injected into another animal of different species, the serum of the latter acquires an 'anti-complement' property. It will be evident, however, from what has been stated above, that when the anti-serum is added to the serum, there are present the three essentials for the deviation phenomenon, viz. (*a*) certain molecules in the serum injected (homologues of receptors), (*b*) the anti-substances to these molecules, and (*c*) complement. Complement will thus be fixed and apparently neutralized. Are there in addition true anti-complements, i. e. anti-substances which unite directly with the haptophore group of complement in the same way as anti-toxin unites with toxin? The possibility of this cannot be excluded, but it is now clear that facts established with regard to anti-

complement action are capable of another explanation. The question must be left an open one and requires fresh investigation in the light of the facts established with regard to deviation (*vide* also p. 39).

Summary of Results

1. A mixture of serum and its anti-serum has the property of fixing or deviating complement and thus interfering with hæmolysis. In this there is a close analogy to the fixation of complements by cell-receptors in association with immune-bodies.

2. A large number of different complements may be fixed by the same combination of serum and anti-serum : some complements, however, may not be fixed.

3. The amount of homologous serum necessary to produce a distinct deviation of complement is extremely small—0·000,01 c.c. and even less : as a rule it is many times less than the amount necessary to give a visible precipitate with the anti-serum.

4. When a precipitate forms, the deviating substance is present in the precipitate and may be so exclusively : precipitation is, however, not essential, as the deviation phenomenon may be given by an anti-serum without the formation of a precipitate.

5. The precipitin and deviation tests give results which are in great part in accord as regards specificity.

6. For any given amount of anti-serum there is an optimum amount of homologous serum which gives maximum deviation of complement : above, as well as below, the optimum the deviation diminishes.

7. The deviation phenomenon produces an effect similar to an ' anti-complement ' action and the views generally held with regard to anti-complements require revision. It is, however, still left an open question whether true anti-complements exist.

PART III

ON THE ANTI-BACTERIAL PROPERTIES OF SERUM

I. OPSONIC ACTION

(*a*) ON THE COMBINING PROPERTIES OF OPSONINS OF NORMAL SERUM

Since the publication of the original paper by Wright and Douglas [1] on opsonins much important work has been done on the subject, and many important facts have been ascertained. The result has been to establish the cardinal part played by opsonic substances in the phenomena of phagocytosis, and also to supply a means of estimating the degree of immunity in one of its aspects. It must be admitted, however, that much doubt still exists as to the biochemical position of the substances in question. Is there, for example, a single type of substance which is thermolabile like bactericidal complement, and which increases during the process of immunization, or are there at least two classes of substances, one of which is thermolabile and another thermostable, and if so, what is the relation of these to each other? Among the phenomena of immunity already known, we have, on the one hand, certain effects produced by what, so far as is known, are single substances, of which agglutinins may be taken as the type; and, on the other hand, effects which are due

[1] Wright and Douglas, *Proc. Roy. Soc.*, London, vol. lxxii, p. 357.

to the co-operation of two substances, namely, immune-bodies and complements.

Further light is still required before we can say to which of these two types the opsonic action conforms, or whether, indeed, it is peculiar to a single class of substances. The work of Bulloch and Atkin [1] tends to show that opsonins are simple substances resembling agglutinins, though differing from them in being more labile, while the results obtained by Leishman [2] and by Dean [3] with regard to immune serums strongly suggest that more than one substance may be concerned, one of which resembles an immune-body. So far, however, as the serums of normal animals are concerned, practically all observers are agreed that the opsonic substance is in most cases destroyed to a vanishing point by heating at a temperature of 55° C. No doubt Dean has shown by a special method that traces of opsonic substance can still be demonstrated in a heated normal serum, but the general fact is that just stated.

In the present research we have endeavoured to obtain an answer to a single definite question, namely, Are the opsonins of normal serum capable of being taken up through the medium of immune-bodies ? The term ' complement ' may be applied to the labile bodies in the serum which are taken up by the complex *receptor* + *immune-body*, without reference to what may be the toxic result of such a combination. Unlike what is seen in the specific combining affinity of immune-body, agglutinin, &c., for the corresponding receptor, the haptophore group of complement shows, as pointed out above, a certain community in its combining affinities. The most diverse combinations of immune-bodies with their corresponding receptors will take up the same complement; also a bacterial receptor + immune-body

[1] Bulloch and Atkins, ibid, vol. lxxiv, p. 379.
[2] Leishman, *Trans. Path. Soc.*, London, 1905.
[3] Dean, *Proc. Roy. Soc.*, London, vol. lxxvi, p. 506.

may take up hæmolytic complement, and a hæmolytic receptor + immune-body may take up bactericidal complement. This statement may be made in a general sense without implying that there may not be multiple complements in a serum ; on the contrary, we know that some complements may not be taken up. So far as the general laws of chemical combination in immunity are concerned, the distinguishing property of complement is that it is absorbed or fixed through the medium of an immune-body ; whether it produces a bactericidal, hæmolytic, &c., effect or not, is in a sense accidental, depending on the susceptibility of the cell to the zymotoxic group. It may be of interest to recall the instance recorded above, in which an agglutinating effect was produced by ' complement ' in this sense. Do the thermolabile opsonins of normal serum belong to the group of complements, or do they conform to anti-substances with specific combining affinity ? In the present paper we leave out of consideration the opsonic substances which can be demonstrated in a heated immune serum and the small residuum which may be present in a heated normal serum. We simply determine what effects on the opsonic power of normal serum are produced by certain methods of absorption which lead to the fixation of hæmolytic and bacteriolytic complements. To what extent is the opsonic action of the serum reduced by these absorptive methods, as compared with the effect of heating the serum at 55° C. ?

In investigating the question as to the absorption of opsonins we have used the immune-bodies corresponding to the receptors of (*a*) red corpuscles, (*b*) blood serum, and (*c*) bacteria. The results are shewn in the three following sections.

I. Absorption by Red Corpuscles Treated with Immune-body (Sensitized Red Corpuscles)

The method here is to introduce red corpuscles combined with multiple doses of immune-body into a normal serum, and then to determine what properties have been removed from the serum. This is most conveniently done by fixing the hæmoglobin of the red corpuscles by heat, at a temperature which does not destroy the receptors—does not, that is, interfere with their powers of absorbing immune-body and thereafter complement. The following are the details :—

Twenty c.c. of a 5 per cent. suspension of ox's corpuscles, washed free of serum, in 0·85 per cent. NaCl solution are taken, and excess of immune-body from a rabbit treated with injections of ox's corpuscles is added. (The corpuscles in question can take up about ten hæmolytic doses of immune-body, so we add rather more than this amount.) After an hour is allowed for combination of the immune-body the corpuscles are centrifugalized and washed in salt solution. They are then placed overnight in a serum sterilizer at 55° C. By such an exposure to this temperature the mixture has been changed into a turbid brownish suspension, the corpuscles being clumped in small granular masses, but the power of absorbing complement is still retained. The mixture is then centrifugalized and the altered corpuscles are washed twice in salt solution. After the last centrifugalization the fluid is removed as completely as possible, and the deposited corpuscles are used for testing their absorbing properties.

The deposit obtained in the above manner is added to 1·5 c.c. of guinea-pig's normal serum, and the mixture is placed in the incubator at 37° C. for one and a half hours. The mixture is then centrifugalized and the serum is pipetted

off; it has become slightly brownish in appearance. We shall speak of such a serum as *treated serum*, and we may compare it as regards hæmolytic, bactericidal and opsonic qualities, with normal guinea-pig's serum, and with the same serum heated at 55° C. for an hour.

1. *Hæmolytic Action of the Three Serums.*

This is tested on 1 c.c. suspension of ox's corpuscles treated with immune-body. The results are :—

The treated serum is practically without effect, 0·2 c.c. giving no lysis.

The heated serum, of course, is likewise without effect.

The normal serum produces complete lysis in a dose of 0·015 c.c.

The treated serum has thus lost its hæmolytic action, or, in other words, the complement concerned in hæmolysis has been removed from it.

2. *Bactericidal Action*

This is tested by the method of Neisser and Wechsberg. A small quantity, $\frac{1}{5000}$ c.c., of a one day's bouillon culture of the bacterium is added to each of a series of tubes along with different amounts of the serum (0·05, 0·1, 0·2 c.c.) and a few drops of bouillon; the mixtures are made up to 1 c.c. with salt solution. The tubes are placed in the incubator for three hours and at the end of that time 0·025 c.c. from each is added to a tube of melted agar and the agar is then plated. The colonies are counted after incubation at 37° C.

(*a*) $\frac{1}{5000}$ c.c. of a bouillon culture of *B. typhosus*.

Amount of Serum.	*Number of Colonies in Plates.*		
	Treated Serum.	*Heated Serum.*	*Normal Serum.*
0·2 c.c.	1,000–2,000	1,000–2,000	0
0·1 c.c.	,,	,,	0
0·05 c.c.	,,	,,	About 60

(*b*) $\frac{1}{5000}$ c.c. of a bouillon culture of *B. dysenteriæ* (Kruse).

Amount of Serum.	*Number of Colonies in Plates.*		
	Treated Serum.	*Heated Serum.*	*Normal Serum.*
0·2 c.c.	Some hundreds	Some hundreds	0
0·1 c.c.	,,	,,	3
0·05 c.c.	,,	,,	Hundreds

It is thus seen that while the normal serum has a marked bactericidal action on both the organisms tested, the treated and the heated serums alike have been deprived of their bactericidal properties. (Controls were made at the same time without serum, and also to test the sterility of the serums.)

3. *Opsonic Action*

This was tested, according to the method of Wright and Douglas, against a suspension of *Staphylococcus aureus*, with the following results :

Average number of cocci ingested per leucocyte

Treated Serum.	*Heated Serum.*	*Normal Serum.*
1·1	1·25	28·5

It thus appears that the serum treated in the manner indicated has practically the same opsonic effect as the heated serum. In the films prepared with both of these serums, one met here and there with single leucocytes containing a considerable number of cocci, but nearly all were quite empty.

The general result of these experiments is to show that the receptors of red corpuscles in union with the corresponding immune-body take up not only 'hæmolytic complement', but also 'bactericidal complement' and 'opsonin' of normal serum.

II. Absorption by Serum Precipitate

It has been now well established by the researches of Moreschi, Gay, and others, including ourselves (*vide* p. 133) that a precipitate produced by a precipitin acting on the homologous serum has the property of fixing various complements. Certain molecules in the serum used for injecting the animal in order to develop the precipitin give rise to anti-molecules, and the combination of the two takes up complement, as is readily shown by hæmolytic tests. The anti-molecules, so far as the fixation of complement is concerned, behave like immune-bodies, though certain differences obtain, as we have already pointed out (p. 157). We have inquired whether this combination of serum-receptors *plus* immune-bodies also absorbs the opsonins of normal serum. The experiments are carried out in a manner analogous to the previous, a serum precipitate being substituted for red corpuscles treated with immune-body.

The precipitate is produced by adding 0·05 c.c. of ox's serum heated at 55° C. to 1·5 c.c. of precipitin (anti-serum produced by injecting a rabbit with ox's serum) in 20 c.c. of 0·85 NaCl solution. The mixture is allowed to stand overnight, and next day the precipitate is separated and washed several times in salt solution. After a final centrifugalization the fluid is removed as completely as possible and 2 c.c. of guinea-pig's normal serum is added to the precipitate. The precipitate is well shaken up in the serum, and the mixture is placed in the incubator for one and a half hours at 37° C. The mixture is then centrifugalized, and the clear serum is pipetted off from the precipitate. As before, the resulting serum will be spoken of as treated serum, and its properties will be compared with those of heated and of normal serums.

1. *Hæmolytic action.*

The treated serum, like the heated serum, has practically no hæmolytic action.

The normal serum produces lysis of the test corpuscles in a dose of 0·015 c.c.

2. *Bactericidal action*, tested on $\frac{1}{2000}$ c.c. of a bouillon culture of *B. dysenteriæ* (Kruse).

Amount of Serum.	*Number of Colonies in Plates.*		
	Treated Serum.	*Heated Serum.*	*Normal Serum.*
0·2 c.c.	Countless	Countless	0
0·1 c.c.	,,	,,	About 20
0·05 c.c.	,,	,,	Thousands

The treated serum has thus lost its bactericidal action.

3. *Opsonic action*, tested on an emulsion of *Staphylococcus aureus.*

Average number of cocci ingested per leucocyte

Treated Serum.	*Heated Serum.*	*Normal Serum.*
0·40	0·48	27·7

The opsonic action of the treated serum is therefore as low as that of the heated serum ; that is, the normal labile opsonin has been absorbed by the serum precipitate.

III. Absorption by Bacteria Treated with Immune-body ('Sensitized Bacteria')

The method is the same as in the previous cases. The bacteria, combined with immune-body, are washed in salt solution and, after the salt solution is pipetted off as completely as possible, are added to fresh guinea-pig's serum. The mixture is placed in the incubator for one and a half hours, and then the bacteria are deposited by centrifugalization and the serum is pipetted off.

In this case there is, however, a complication, inasmuch as the bacteria alone absorb a certain amount of complement. We accordingly treat another sample of serum *with bacteria only*, this being done in exactly the same way, with the single difference that the bacteria are not previously treated with immune-body.

The following experiment is an example of the different powers of absorbing hæmolytic complement possessed by treated (sensitized) and untreated bacteria respectively. A test of this kind was always employed before their effect on opsonin was estimated.

Three series of tubes are prepared. Each receives 0·05 c.c. of emulsion of a culture of *B. coli*.

To the first series (*a*) no anti-serum is added.

To the second series (*b*) 0·001 c.c. anti-serum is added.

To the third series (*c*) 0·01 c.c. anti-serum is added.

To the several tubes in series increasing quantities of guinea-pig's complement are added. The contents are made up to 1 c.c. with salt solution, and the tubes are placed in the incubator for one and a half hours to allow combination of complement to occur. To each tube 1 c.c. of a suspension of ox's corpuscles (sensitized) is added to test for free complement. Complete lysis is got in the different series with the following amounts of added complement :—

Emulsion alone . . . 0·03 c.c.

Emulsion + 0·001 c.c. anti-serum, 0·075 c.c.

Emulsion + 0·01 c.c. ,, 0·125 c.c. gives ¾ lysis.

The hæmolytic dose of untreated complement was 0·015 c.c.

It is thus shown that, while the bacteria alone take up a certain amount of complement, the addition of immune-body (anti-serum) leads to the taking up of much more. How is the opsonic effect influenced ? The following examples show this clearly.

We shall call the serum treated with bacteria + immune-body Serum A, and the serum treated by bacteria alone Serum B.

Anti-serum to B. coli

Treated Serum A = 1 c.c. of guinea-pig's serum treated with 0·25 c.c. emulsion of *B. coli* + 0·05 c.c. anti-serum (immune-body).

Treated Serum B = 1 c.c. of guinea-pig's serum treated with 0·25 c.c. emulsion of *B. coli* alone.

The organisms were killed by heat before being used.

1. *Hæmolytic Action.*

Treated Serum A.	*Treated Serum B.*	*Normal Serum.*
Practically without hæmolytic effect	Hæmolytic dose = 0·075 c.c.	Hæmolytic dose = 0·015 c.c.

2. *Opsonic action,* tested on emulsion of *Staphylococcus aureus.*

Average number of cocci ingested per leucocyte

Treated Serum A.	*Treated Serum B.*	*Normal Serum.*
0·5	26·2	46·7

It is thus seen that treated serum A is practically without hæmolytic and opsonic action. Treated serum B occupies an intermediate position, and its hæmolytic action appears to be more reduced than its opsonic action. This is probably, however, due to there being excess of opsonin in the normal serum, so that a considerable reduction of the quantity might be produced without there being a marked effect on the number of cocci ingested.

The following is another example, guinea-pig's serum being treated as above with *B. coli* + its anti-serum, and with the bacillus alone :—

1. *Hæmolytic action,* shown by the minimum hæmolytic dose.

Treated Serum A.	*Treated Serum B.*	*Normal Serum.*
0·3	0·075	0·02

2. *Opsonic action.*

Average number of cocci ingested per leucocyte—two different suspensions (a) and (b).

Treated Serum A.	*Treated Serum B.*	*Normal Serum.*
(*a*) 2·85 (*b*) 0·6	26·6 1·31	51·2 8·06

In this experiment treated serum A has still traces of opsonic and hæmolytic action, but both properties are much more reduced than in serum B.

The following is another example where the normal guinea-pig's serum was treated with *Spirillum Metchnikovi* + its anti-serum, and with the spirillum alone :—

1. *Hæmolytic action.*

Minimum Hæmolytic Doses

Treated Serum A.	*Treated Serum B.*	*Normal Serum.*
0·12	0·05	0·01

2. *Opsonic action.*

Number of cocci ingested per leucocyte

Treated Serum A.	*Treated Serum B.*	*Normal Serum.*
2·8	6·1	31

The hæmolytic action of Serum A is less reduced than in the previous examples, and the opsonic action is also relatively more marked.

3. *Bactericidal action*, tested on $\frac{1}{5000}$ c.c. of bouillon culture of *B. dysenteriæ.*

Number of Colonies

Amount of Serum.	*Treated Serum A.*	*Treated Serum B.*	*Normal Serum.*
0·2 c.c.	1,000–2,000	1,000–2,000	5
0·1 c.c.	more	more	800
0·05 c.c.	thousands	thousands	1,000–2,000

Both of the treated serums have been practically deprived of their bactericidal power, though there is evidence of a small residuum. The colonies appeared to be approximately the same in the two series but were too numerous to estimate accurately.

The marked reduction of bactericidal action resulting from treatment with a heterologous organism (V. Metchnikovi) is in accordance with the results to be recorded later (p. 201).

It is thus brought out that a bacterium combined with the homologous immune-body absorbs or fixes bactericidal complement, hæmolytic complement, and normal opsonin alike. We have never been able to obtain a diminution of one of these without the other two being similarly affected, though further observations will be necessary before a general statement can be made. It is to be noted that these results are obtained with *powerful* complement-absorbers. When small quantities of bacterial emulsions are used there may be a much greater diminution in bactericidal than in hæmolytic action (p. 202).

It will be noticed on comparing the results in the above tables that there is remarkable similarity in the variation of the hæmolytic action and of opsonic actions, produced by the methods used. Diminution in the opsonic effect associated with moderate fall in hæmolytic power is best seen when the opsonic index of the normal serum comes out low. This is in accordance with what is seen on diluting a normal serum with high opsonic power, the effects of further diluting being most marked after the opsonic index has been reduced somewhat by previous dilutions.

General Results

We have thus tested the three chief varieties of immune-bodies ('amboceptors'), namely, those obtained by the injection of (*a*) red corpuscles, (*b*) serum, and (*c*) bacteria, respectively, and have found that in each case the combination of receptor + immune-body removes the opsonin of normal serum as tested by an emulsion of *Staphylococcus aureus*. We have also shown that a bacterium treated with immune-body takes up more of the normal opsonin than the same bacterium untreated, just as it takes up more of the normal complement as tested by hæmolysis. If we define a complement from the chemical point of view as above explained, it is evident that the thermolabile opsonins

of normal serum belong to the group of complements (alexines). As shown above, we found a striking resemblance in the diminution of hæmolytic, bactericidal and opsonic action produced by the different methods of absorption, but at present we pronounce no opinion as to the identity or non-identity of the substances producing these effects. Much remains to be done ere the relation of opsonins to other bodies in serum can be definitely assigned, but the above results are very definite and of a striking nature. We shall consider in the next section whether the comparatively thermostable opsonin which may be present in an immune serum can be removed by the methods of absorption employed above. In this way we hope to gain some light on the question as to whether there are one or two classes of opsonins, so far as their combining relationships are concerned.

Note.—It is to be noted that these observations refer only to the labile substance of the serum, which is destroyed at 60° C. and without which the opsonic action of a normal serum is practically nil. They leave out of consideration the question whether a small quantity of stable substance of the nature of an immune-body is also concerned. The results obtained have been confirmed by others, e.g. by Neufeld and Hüne,[1] by Levaditi[2] and his co-workers. An interesting additional confirmation has also recently been published by Browning.[3] Sachs and Teruuchi had previously shown that if a normal serum were diluted with five volumes of distilled water and kept at 37° C. for an hour and a half, then on restoring the original salt content the hæmolytic complement was found to be destroyed. Browning found that by the same procedure the complement opsonin of normal serum was also destroyed, whereas the immune opsonin (*vide infra*) was unaffected.

[1] Neufeld and Hüne, *Arbeit. a. d. Kais. Ges.-Amt*, 1907, Bd. 25.
[2] Levaditi, various papers in *Compt. rend. Soc. Biol.*, 1907.
[3] Browning, *Journ. Med. Research*, vol. xix, 1908, p. 201.

(*b*) ON THE COMBINING PROPERTIES OF THE OPSONIN OF AN IMMUNE SERUM

In the previous section we have studied the combining properties of the thermolabile opsonins of normal sera, and have shown that various substances or combinations of substances which absorb serum complements also absorb the opsonins in question. Of special interest is the fact that red corpuscles, and also heated normal sera, which by themselves have no appreciable effect either on complements or on opsonins, absorb or fix both of these bodies when combined with their corresponding anti-substances, immune-bodies and precipitins respectively. Since these results were published we have extended our experiments, and have always obtained the same result—the opsonin is always fixed when the complement is fixed. Using the term complement in the bio-chemical sense, we have said that the normal thermolabile opsonins belong to the group of complements. And we have also found that just as complements in their combining affinities do not possess specific properties, but are absorbed by a great many different substances, so also these thermolabile opsonins show a corresponding community in their combining relationships. We, however, expressly left out of consideration (*a*) the special opsonins of immune sera, and also (*b*) the thermostable opsonins of normal sera, of which latter the stable opsonin of human serum for the diphtheria bacillus may be taken as an example. In the present communication we shall consider the thermostable opsonins of immune sera.

It is unnecessary to enter in detail into the literature of the subject, as this has been already done by Dean.[1] It is, however, advisable for the sake of clearness to refer to the chief facts which have been established and to the chief

[1] Dean, *Roy. Soc. Proc.*, vol. lxxvi, p. 515.

points at issue. It is now a considerable time since Metchnikoff showed that the establishment of active immunity towards various bacteria was often accompanied by increased phagocytic action on the part of leucocytes and other cells, or by the appearance of phagocytic action when this was absent under natural conditions. Denys and Leclef[1] showed in the case of rabbits immunized against streptococci that the increased phagocytosis was due not to changes induced in the leucocytes, but to an alteration in the serum, and pointed out that the leucocytes of the immune animal when placed in a normal serum showed no greater phagocytic activity than normal leucocytes did. Wright and Douglas[2] were the first to show that phagocytosis by leucocytes in the presence of normal serum depended upon certain thermolabile substances—' opsonins '—in the serum which became fixed to the bacteria in question and made them a prey to the leucocytes. Their results were confirmed by Bulloch and Atkin,[3] by Hektoen and Ruediger,[4] and by others, and may now be accepted as established beyond question. We have, on the other hand, a large group of observations which show that in *immune sera* the substance which leads to the phagocytosis of bacteria, or of red corpuscles, as the case may be, is thermostable, i. e. resists a temperature of 55° C. for an hour. Among such observations may be mentioned those of Savtchenko,[5] Neufeld and Rimpau,[6] Dean,[7] Leishman,[8] and others. And Wright and Reid[9] have shown that in certain cases the serum of patients suffering from tuberculosis may contain a considerable proportion of

[1] Denys and Leclef, *La Cellule*, 1895, p. 177.
[2] Wright and Douglas, *Roy. Soc. Proc.*, vol. lxxii, p. 357.
[3] Bulloch and Atkin, *Roy. Soc. Proc.*, vol. lxxiv, p. 379.
[4] Hektoen and Ruediger, *Journ. of Infectious Diseases*, 1905, p. 128.
[5] Savtchenko, *Annales de l'Inst. Pasteur*, 1902, p. 106.
[6] Neufeld and Rimpau, *Deutsch. Med. Wochenschr.*, 1904, p. 1458.
[7] Dean, *op. cit.* [8] Leishman, *Path. Soc. Trans.*, London, 1905.
[9] Wright and Reid, *Roy. Soc. Proc.*, vol. lxxvii, p. 211.

heat-resisting opsonin, a circumstance which may aid the diagnosis. The question therefore arises as to what is the relationship between the thermolabile and the thermostable opsonins. Dean thinks that the opsonin is of the nature of an immune substance, and that in the case of normal serum it undergoes a large fractional destruction by heat, whereas in an immune serum the portion which is thermostable is increased. He, however, mentions the possibility that the opsonic effect of a normal serum may in part be due to complement.[1] Wright and Reid consider that the opsonin in a normal and in an immune serum alike is one and the same, and prefer to call it thermolabile. The question as to the identity of such substances may be studied in other ways than that of testing their powers of resistance to heat. One method is to test whether cell-receptors combined with their corresponding immune-bodies will take up the opsonins of immune sera, as we have shown that they take up the labile opsonins of normal sera and as they take up complements. Another method is to test the degree of specificity in their combining affinities—to test, that is, to what extent one bacterium will absorb the opsonins for other bacteria. This has been done by Bulloch and Western[2] to a certain extent, and also by Hektoen[3] in the case of hæmopsonins, and to these experiments further reference will be made below. But the method has not been carried out fully in a comparative way as between normal and immune sera respectively.

We shall now give an account of experiments performed by us to elucidate these questions in the case of an immune

[1] In his more recent publications he expresses definitely the opinion that this is the case, and considers that the opsonic action of a normal and immune serum alike resembles hæmolytic action in being due to the co-operation of immune-body (present in traces in normal serum) and of complement.

[2] Bulloch and Western, *Roy. Soc. Proc.*, vol. lxxvii, p. 531.

[3] Hektoen, *Journ. of Infectious Diseases*, 1906, p. 721.

serum, giving in each instance for comparison the effects on normal opsonins under the same conditions of experiment. We have made use of two samples of anti-staphylococcic serum. One of these was kindly given to us by Dr. Dean, for which we have pleasure in recording our indebtedness, whilst the other was obtained by us from a rabbit by repeated intravenous injections of dead cultures of *Staphylococcus aureus*. As it was employed at various stages in the process of immunization, the degree of the opsonic effect varies considerably in the different experiments. We may state that heating for several hours at 55° C. appears to have no appreciable effect on the thermostable immune opsonin. The serum thus contrasts very markedly with the normal rabbit's serum, the opsonic effect of which is practically destroyed by heating for an hour at 55° C.

I. The Effects of Absorbers of Complement on Normal and Immune Opsonins

We have tested whether or not the three combinations of receptors + immune-body used in the previous experiments (p. 161) absorb the opsonin of the immune serum.

The two following experiments bring out at a glance the differences in the effects of treating a normal and an immune serum in various ways, each serum being afterwards tested on an emulsion of the *Staphylococcus aureus* in the usual way :—

Normal serum of rabbit—	Opsonic count[1]
Fresh and untreated	18·1
Heated one hour at 55° C.	0·6
Unheated and treated with red corpuscles + immune-body	0·7
Unheated and treated with serum precipitate	1·0
Unheated and treated with emulsion of *Staphylococcus aureus*	0·7

[1] This, of course, means the average number of cocci ingested per polymorphonuclear leucocyte in Wright's technique. The observations given in each table were of course carried out at the same time.

It is thus seen, in accordance with our previous results, that practically all the normal opsonin is removed by these various methods of treating the serum. To contrast with this we give the results in the case of an immune serum.

	Opsonic count
Anti-staphylococcus serum from rabbit—	
Fresh and unheated	12·3
Heated one hour at 55° C.	2·9
Unheated and treated with red corpuscles + immune-body	3·1
Unheated and treated with serum precipitate	3·5
Unheated and treated with emulsion of *Staphylococcus aureus*	0·7

It is thus seen that[1] treating the serum with the substances which absorb complements has practically the same effect on the serum as heating has; there being approximately the same amount of immune opsonin left, the latter being unaffected by the complement-absorbers. An emulsion of staphylococcus, however, removes the opsonin almost entirely.

Other confirmatory examples may be given :—

Treatment by Precipitate

	Opsonic count
Anti-staphylococcus serum—	
Fresh and unheated	18·2
Heated one hour at 55° C.	6·9
Unheated and treated with precipitate	5·8

In this case there was an enormous excess of precipitate, the actual bulk of the precipitate being three times that of the serum; there would, therefore, probably be some dilution of the serum. The treated serum has practically the same opsonic value as the heated; that is, in spite of the large amount of absorber (precipitate) the immune opsonin is unaffected.

[1] The methods of treating a given serum are the same as those described by us in the previous section (p. 162).

Treatment by Precipitate[1]

	Opsonic count
Anti-staphylococcus serum—	
Heated at 55° C.	7·2
Heated and treated with precipitate	7·0
Normal serum—	
Unheated	24·6
Unheated and treated	0·86

Treatment by Red Corpuscles + Immune-Body

	Opsonic count
Anti-staphylococcus serum—	
Heated at 55° C.	8·3
Heated and treated	9·4
Normal serum—	
Unheated	20·8
Unheated and treated	0·8

The thermolabile opsonin of the normal serum is absorbed, whereas the thermostable opsonin of the immune serum is unaffected.

Treatment by Emulsion of V. Metchnikovi *+ Immune-body*

	Opsonic count
Anti-staphylococcus serum—	
Heated at 55° C.	17·2
Heated and treated	17·5
Normal serum—	
Unheated	25·0
Unheated and treated	2·36

The result is the same as before.

We have also endeavoured to remove the opsonin of the immune serum by treating it twice with a considerable quantity of serum precipitate, the precipitate being separated by centrifugalization after each treatment, but no diminution of the opsonin has resulted. The following is

[1] In this and the two following experiments the anti-serum was the one which we obtained from Dr. Dean.

an example in which the effects on the normal and the immune serum of the rabbit are once more compared :—

Normal Serum

	Opsonic count
Normal serum of rabbit (unheated)	17·0
„ „ treated once with staphylococcus emulsion	1·6
„ „ „ twice „ „	0·25
„ „ „ once with serum precipitate . .	11·3
„ „ „ twice „ „	0·3

Immune Serum

Anti-staphylococcus serum (heated at 55° C.)	23·3
„ „ treated once with staphylococcus emulsion	23·0
„ „ „ twice „ „	2·2
„ „ „ once with precipitate . .	25·0
„ „ „ twice „ . . .	23·0

The result, again, is that the serum precipitate removes the opsonin from the normal serum, but has no effect on the stable opsonin of the immune serum.

Summary.—It appears, from the above experiments, that in the case of a fresh anti-staphylococcus serum the effect of treating with substances which absorb complement is practically the same as heating the serum, there being a considerable residuum of opsonin in both cases. Further, after the thermolabile opsonin has been destroyed by heat, the stable opsonin remains practically unaffected by treatment which removes normal complement and normal opsonin. In other words, *substances which we may call complement-absorbers do not fix or combine with the thermostable opsonin of the immune serum.*

II. On the Relative Specificity of Normal and Immune Opsonins

An emulsion of dead bacteria is well known to have the power of absorbing serum complements, as shown, for example, by hæmolytic tests ; it also absorbs the opsonin of

a normal serum as tested by the *Staphylococcus aureus*. We have accordingly tested the effect on the opsonin of our immune serum. In the following tables the comparative results are shown :—

Normal Serum

	Opsonic count
Normal serum of rabbit (unheated)	38·0
" " treated with *Staphylococcus aureus*	0·02
" " " *Bacillus coli* . . .	3·3
" " " *V. Metchnikovi* . .	4·11
" " " *Bacillus tuberculosis* .	2·6
" " " *Bacillus pyocyaneus* .	3·4

Immune Serum

	Opsonic count
Anti-staphylococcus serum (heated at 55° C.) . . .	7·8
" " treated with *Staphylococcus aureus*	0·36
" " " *Bacillus coli* . .	8·8
" " " *V. Metchnikovi* . .	7·5
" " " *Bacillus tuberculosis* .	7·9
" " " *Bacillus pyocyaneus* .	6·7

The tables show that emulsions of all the organisms tested produce a marked diminution of the normal opsonin, whereas none of them, with the exception of the staphylococcus, have any appreciable effect on the immune opsonin.

In another experiment we treated the heated immune serum twice with large quantities of *B. coli*, *B. dysenteriæ*, *Cholera spirillum*, *B. typhosus*, *Staphylococcus pyogenes albus*. In the case of the last mentioned there was apparently a slight diminution of the opsonic power, viz. from 26·7 to 20·3 —a comparatively trifling effect in view of the close relationships of the two organisms; the untreated serum was, however, practically without opsonic effect on the *Staphylococcus albus*. In the case of the other bacteria mentioned, there was no diminution whatever of the opsonin after treatment with the organisms.

These experiments, by the method of absorption, bring

out a very marked difference as regards specificity between the opsonins of a normal and of an immune serum. *With the exception of the possible slight effect in the case of the Staphylococcus albus, we have failed to find any organism, except the Staphylococcus aureus, which absorbs the opsonin from the heated anti-staphylococcus serum.* In the case of the opsonins of a normal unheated serum, the result is entirely different, as is shown by the table given above. Every organism tested has absorbed large quantities of opsonin when the *Staphylococcus aureus* is used as the test for opsonic action.[1] At the same time, the staphylococcus appears to remove this opsonin more quickly than any of the others. We have tested to what extent it is possible to remove the opsonin of normal serum for *Staphylococcus aureus* by treating the serum twice with an emulsion of another organism, e.g. the *B. coli*. The results are :—

Normal Serum

	Opsonic count
Guinea-pig's serum (unheated)	21·5
,, ,, treated once with *Staphylococcus aureus*	1·0
,, ,, ,, twice ,, ,,	0·4
,, ,, ,, once with *B. coli* . . .	2·1
,, ,, ,, twice ,, . . .	1·3

There is, therefore, only a slight difference as regards absorbing powers in favour of the *Staphylococcus aureus*.

The immune serum was tested at the same time, and once more a great difference was brought out.

Immune Serum

	Opsonic count
Anti-staphylococcus serum (heated at 55° C.) . . .	26·7
,, ,, treated twice with *B. coli* . .	25·4
,, ,, ,, *Staphylococcus aureus*	1·6

[1] These results, so far as the normal opsonins are concerned, are in harmony with those of Simon, Lamar, and Bispham, whose paper (*Journ. of Exper. Med.*, December, 1906, p. 651) came into our hands after our original communication was written.

These results, as regards the specificity of the normal opsonins, appear to be at variance with the results obtained by Bulloch and Western. They found only a slight reduction of the tubercle opsonin of normal human serum on treatment with the *Staphylococcus aureus*, and of the staphylococcus opsonin on treatment with the tubercle bacillus. As stated above, we found a great reduction of the staphylococcus opsonin on treating the normal rabbit's serum with the tubercle bacillus. The difference in the results probably depends upon the amount of the bacterial emulsion employed. In every case we used a large amount ; in the tubes after centrifugalization the volume of the deposit of bacteria would be about a tenth of the volume of the serum, sometimes more. This is, no doubt, a large quantity, but it is to be noted that the same amounts were employed in the case of the immune serum, and no diminution of the opsonin was observed. The difference in the two kinds of serum is, therefore, very remarkable.

We have stated above that the opsonin for a particular organism appears to be more rapidly removed from a normal serum by an emulsion of that organism than of any other, whilst at the same time any bacterial emulsion will absorb large quantities of that opsonin. At present we are unable to give the explanation of that fact. We use the term ' normal opsonin ' for the labile substance which is destroyed by heat, but we do not know whether or not another substance is present in small amount which acts as an immune-body. The problem is very much the same as in the case of bactericidal action. A closely analogous phenomenon was demonstrated by Bordet[1] in the case of hæmolysis. Normal guinea-pig's serum, after treatment with rabbit's corpuscles, is deprived of its lytic action for these corpuscles ; it still, however, produces lysis of pigeon's corpuscles. If, however, the serum be treated by a more powerful absorber of

[1] Bordet, *Annales de l'Inst. Pasteur*, 1901, p. 317.

complement, viz. rabbit's corpuscles treated with immune-body, it loses also its hæmolytic action on pigeon's corpuscles. And it has been shown in the previous section that an emulsion of a bacterium treated with immune-body absorbs more opsonin than the untreated emulsion, the amount of opsonin left being practically the same as when the serum is heated. The following is an additional example :—

Opsonic Action of Normal Human Serum on the Tubercle Bacillus

	Opsonic count
Normal serum unheated	6·5
„ „ heated at 55° C.	0·2
„ „ treated with emulsion of *V. Metchnikovi* + immune-body	0·19

III. The Relations of the Opsonin of an Immune Serum

Our results, as above detailed, show that the opsonin of the anti-staphylococcus serum corresponds as regards specificity and combining relationships with true *anti-substances* developed in the process of immunization. Does it correspond with the type of an immune-body or of an agglutinin ? Does it in combination with its corresponding receptor lead to the absorption of complement, or does it not ? In the first place, it is certain that every immune-body does not produce an opsonic effect. We have an anti-coli serum, for example, which contains immune-body in considerable quantity, but it has no opsonic effect. A given amount of emulsion of the *B. coli* alone was found to take up 0·04 c.c. of guinea-pig's complement, while the same amount of emulsion treated with the anti-serum took up 0·12 c.c., the absorption of complement being tested by the usual hæmolytic methods. In the case of the anti-staphylococcic serum the amount of immune-body appeared to be considerably less. The sample with the greatest

opsonic power gave the following results:—emulsion of staphylococci alone took up 0·03 c.c. of guinea-pig's complement, the same quantity treated with immune-body took up 0·075 c.c. This sample had a very powerful opsonic action, giving, when heated, an average count of 20 cocci per leucocyte in the usual test. The anti-staphylococcus serum which we got from Dr. Dean had a weaker though still very decided opsonic action, yet it produced a scarcely perceptible increase in the amount of complement absorbed; that is, it contained a mere trace of immune-body.[1] We are inclined to think from these results that the opsonin in an immune serum may not be an immune-body, but has the constitution of an agglutinin. But this is merely an expression of opinion, as we know nothing with regard to the real amounts of these substances. In order to say that the immune-body did not act as opsonin, we would need to get an anti-serum with opsonic effect but containing no immune-body, but this we have not yet obtained.

Note.—It is to be noted that in speaking of the opsonin of an immune serum we mean the thermostable anti-substance which by itself has an opsonic effect; the question is as to whether or not this is simply an immune-body. That an immune-body may increase the opsonic effect of an *unheated* immune serum, by leading to the absorption of more complement opsonin, follows from what has been stated above (p. 167). But this is, of course, another matter (*vide* p. 188).

If the opsonin has the constitution of an agglutinin, that is, possesses merely a combining and an active (agglutinating) group, is the opsonin merely the agglutinin? To this question we can give no definite answer. We have tested

[1] In a third anti-staphylococcus serum which we have recently examined, a considerable amount of immune-body, as shown by absorption of complement, was present, yet its opsonic effect was not superior to that of the other two sera.

three anti-sera, viz. to the *Staphylococcus aureus*, the *B. coli*, and the *Vibrio Metchnikovi*, and have found that they all possess agglutinating properties, while the anti-coli serum alone has no opsonic action. It is quite possible that the same substances may act at the same time as agglutinin and as opsonin, and that one of these properties may be wanting in any given case ; but at present we have no facts to justify any expression of opinion.

So far as phagocytosis is concerned, the all-important factor in active immunity would appear to be the development of an immune opsonin with comparatively specific characters. In testing the opsonic effect of an unheated immune serum the result will represent the sum of the actions of the complement-like opsonin and the immune opsonin. As complements do not appear to increase in amount during immunization, a rise of opsonic index will probably depend upon the development of immune opsonin, and will thus have a certain specific character. Thus Bulloch and Western found that inoculation of a patient with tubercle vaccine produced an increase of tubercle opsonin but not of staphylococcus opsonin, and conversely inoculation with staphylococcus vaccine caused an increase of the staphylococcus opsonin but not of the tubercle opsonin. This appears to be in harmony with the results which we have obtained. On the other hand, a fall in the opsonic index might be due to diminution of the complement-like opsonin, and thus be without specific significance. Further observations will be made on this point.

General Conclusions

1. The thermolabile opsonin of a normal serum and the thermostable opsonin of an immune serum are two distinct classes of substances. In addition to differing markedly as regards their resistance to heat, they differ in their combining relationships.

2. The thermostable opsonin of the anti-serum investigated is a true anti-substance, and possesses the comparatively specific characters of anti-substances in general; it is left undetermined whether it has the constitution of an agglutinin or of an immune-body, though certain facts point in favour of the former.

3. Emulsions of other organisms than the organism used in immunization (*Staphylococcus aureus*) do not absorb the immune opsonin; on the other hand, they absorb large amounts of the normal complement-like opsonin.

4. Powerful complement-absorbers—red corpuscles or bacteria treated with immune-body, or serum precipitate—have no effect on the thermostable immune opsonin, whereas they remove almost completely the labile opsonin of the normal and the immune serum alike.

Addendum

Although the more important practical points appear to us to have been brought out by the investigations above detailed, some theoretical questions with regard to opsonic action may be referred to. Attempts have been made to explain all the opsonic phenomena as conforming with the scheme of hæmolysis, according to which hæmolysis by a natural serum depends upon a natural immune-body in association with complement, and hæmolysis by an immune serum depends upon an artificially developed immune-body in association with complement, the immune-body itself having no effect. In a corresponding manner the opsonic action by a normal serum would be due to a natural immune-body acting along with complement opsonin, and the opsonic action of an immune serum would be due to an artificially developed immune-body also acting in association with complement. Now there are a certain number of facts which support such a view. There is, in the first place, the fact that the normal serum may lose a considerable amount

of opsonic effect for a particular organism when treated with an emulsion of that organism at 0° C., as was shown by Bulloch and Atkin, and this might be due to the absorption of a natural immune-body at the low temperature. Dean in his first paper also showed that, in the case of the *staphylococcus aureus*, there remains in human serum after heating at 55° C. a small quantity of thermostable substance which has opsonic effect. On the other hand, there is evidence that normal thermolabile opsonin unites directly with bacteria. The fact that the normal opsonin for the one organism can be removed by treatment with emulsions of other organisms, provided a sufficient quantity be used, shows this; unless we are to assume that for all these organisms there are present sufficient normal immune-bodies to bring the whole of the complement into combination. For example, an emulsion of *B. coli* removes the normal opsonin for *staphylococcus aureus*. If this depends on a natural immune-body for bacillus coli the molecules of this immune-body must at least be in a number to correspond with the whole complement content of the serum, and ascertained facts as to the manner in which complement-opsonin is taken up by untreated and by sensitized bacteria respectively, are against such an assumption (p. 167); in fact the only conclusion possible seems to be that there is not sufficient natural immune-body in a normal serum to correspond with the amount of complement opsonin, but if we add a sufficient amount of bacteria the organisms can remove the complement opsonin by direct combination. This question is discussed further in connexion with bactericidal action (p. 203). If then the normal complement opsonin combines directly with organisms we must believe that it has opsonic action. It would be unjustifiable to suppose that it is without action unless it is brought into combination through the medium of an immune-body. This subject is also referred to in connexion with the

bactericidal action of normal serum. Thus, while we admit that in certain cases a substance behaving like a natural immune-body may be concerned in normal opsonic action, we hold that an opsonic effect on an organism may be due to the direct union of labile opsonin as above explained.

We have next to consider the question of the opsonic action of an immune serum. Its increased action as compared with normal serum is due, as already shown, to the combined action of the specially developed anti-substances acting along with the normal complement-like opsonin, the latter not increasing during the process of immunization.

The question is as to the nature of the co-operation of the two substances. Now it is to be noted, in the first place, that the presence of an immune-body of the ordinary type might lead to increased opsonic effect, as it has been shown above that such an immune-body leads to increased absorption of normal complement opsonin; a greatly increased amount of complement *per bacterium* is brought into union. And Browning has shown in one case that increased opsonic effect is got when organisms are treated first with immune-body and then with complement, whereas on reversing the order of treatment no such augmented effect is obtained. This, however, is not the whole matter, as the anti-body which we have called 'immune opsonin' produces the opsonic effect *by itself*—produces, that is, an effect similar to that brought about by normal complement. To this we have no analogy in the case of hæmolysis or of bactericidal action. That the increase in the opsonic action of fresh immune serum is in part due to this independent action of immune opsonin there can be no doubt. There remains, however, a further point, namely, whether the molecules in the anti-serum which act as immune opsonins have also the constitution of immune-

bodies. If this were so, then the increased opsonic action of a fresh immune serum might be due to the following factors :—

(*a*) Immune-body (amboceptor) leading to increased combination of complement opsonin ;

(*b*) Immune opsonin producing opsonic effect by itself ;

(*c*) The same molecules in the anti-serum acting both as immune-bodies and as immune opsonins.

We consider it proved by what has been stated above that both of the two first possibilities hold good. With regard to the third there is still doubt. In a serum where there is a mixture of anti-substances it is a very difficult matter to demonstrate that immune opsonins act at the same time as immune-bodies. If this were so, then we would have a new type of anti-substance ; one which has a zymotoxic group and one which at the same time leads to the absorption of complement. Theoretically there is nothing against such a supposition. In fact it is suggested by the deviation of complement brought about by a precipitin and the homologous serum. We are, however, not justified in saying that all the molecules of immune opsonin have the constitution of immune-body. The fact above referred to, namely :—that an immune serum may have marked opsonic action and contain comparatively little immune-body, speaks against such a supposition. It is quite likely that the immune opsonins, in part at least, have the constitution of agglutinins, possessing a zymotoxic group, but not leading to the union of complement. In any case they are bodies of great importance in immunity as they can act in the absence of complement.

The term ' immune opsonin,' as used above, has practically the same significance as the ' bacterio-tropin ' of Neufeld ; that is, it denotes a specific thermostable anti-substance which, by itself, promotes phagocytosis. This

writer, in a recent review[1] of the subject, restricts the term 'opsonin' to cases where the thermolabile complement opsonin is concerned, and considers such opsonic action always to proceed according to the scheme of immune-body (natural or artificial) + complement. It is quite evident that the term 'opsonin' as signifying a body which promotes phagocytosis cannot be applied to any one substance; it can only be used as signifying a property of a substance, not its constitution. We, however, prefer the term 'immune opsonin' to 'bacterio-tropin,' as it is sufficiently definite and the term 'opsonin' is so convenient and now so widely understood. Neufeld discusses the relation of the bacterio-tropins (immune opsonins) to immune-bodies, and concludes that they are distinct substances. He, however, appears to rely too much on the definition of immune-body. If this definition includes *two* properties as necessary, namely, (*a*) that it leads to the union of complement; and (*b*) that it produces no recognizable effect by itself; then manifestly bacterio-tropins are not immune-bodies, as they produce such an effect. (We agree with him in holding that the opsonic action of a bacterio-tropin is not due to complement supplied by leucocytes.) But the important question still remains, namely, whether the same molecule which produces an opsonic effect, by means of its zymotoxic group, has also the property of an immune-body of leading to the union of complement. With regard to this, all that we can say is that, though it is quite probable, we do not consider that it has yet been satisfactorily demonstrated. Proof is equally wanting that all the molecules of immune opsonin also have the property of immune-body referred to. The complexity of the question becomes still more evident when we consider that immune-bodies do not always lead to the fixation of complement; this, as has been shown above,

[1] Neufeld, '*Kolle und Wassermann's Handbuch der pathogenen Mikro-organismen*,' Ergänzungsband II, Heft ii, p. 303.

depends both upon the receptors to which the immune-body is attached and also upon the complement.

A further question arises with regard to cases where the labile complement opsonin is concerned, namely, as to whether or not the substances producing opsonic effect are the same as those which bring about bactericidal action or bacteriolysis. The statement is not infrequently made that, because a serum may produce opsonic effect and no bactericidal action, therefore the substances concerned in these two actions are different. This line of reasoning is quite fallacious, as no conclusion can be drawn when the two effects are tested on two different organisms. The same substance may quite well produce the bactericidal action on one organism and no bactericidal action on another organism, and yet be capable of producing the change in the latter necessary for opsonic effect. The process of opsonization necessarily means a much less disruptive effect than the change leading to death of the organism ; it may mean nothing more than this, or it may be different in kind. As a matter of fact, organisms may take up bactericidal complement, and when treated with the homologous immune-body may take it up in large amount, without any bactericidal effect following, simply because the organism is not sufficiently susceptible to the toxic action of the combined complement. The identity or non-identity of the substances concerned in bactericidal and in opsonic action when complement is concerned, must be left an open question for the present.

Our general conclusions are that in the case of normal sera the opsonic effect is generally due to the labile non-specific complement ; it may act with or without a natural immune-body. (A close parallelism as regards mechanism may be drawn between this and normal bactericidal action, and the same principles will probably be found to obtain (p. 203).) In the case of immune sera the opsonic effect

may be increased by immune-bodies leading to the union of more complement. There is in addition, however, the formation of immune opsonin (bacterio-tropin), which produces an opsonic effect by itself. This substance has accordingly the constitution of an agglutinin ; it is left an open question whether, and if so, to what extent, it may lead to the union of complement that is functionate also as an immune-body.

II

ON THE BACTERICIDAL ACTION OF NORMAL SERUM

The work which has been carried out with regard to the bactericidal action of normal serum may be said to fall into two chief periods. The first starts from the original demonstration of this property by Nuttall in 1888, a demonstration which may be said to constitute the foundation of the subsequent researches on the serum in relation to immunity. That bactericidal action was sometimes possessed by the serum of a naturally immune animal, and absent in the case of a susceptible animal, whereas in other instances this did not hold; that immunity could not be explained by the presence or absence of this property alone; that the bactericidal substance was relatively labile, being destroyed as a rule at 55° C.; and that bactericidal action *in vitro* did not always correspond with bactericidal action *in vivo*—these were some of the most important facts subsequently established. The second period dates from Pfeiffer's demonstration of the dual constitution of an anti-bacterial (bacteriolytic) serum—the presence of the comparatively stable specific substance, developed during the process of immunization, and the labile substance present in the normal animal. It is unnecessary to refer to the subsequent researches of Metchnikoff, Bordet, Ehrlich and Morgenroth, v. Dungern, and others, which have supplied the additional fundamental facts with regard to immune-bodies and complements in the case of bacteriolytic and hæmolytic sera alike, as these are now sufficiently well known. The fact that in the case of the hæmolytic action of a normal serum

on foreign corpuscles the lysin concerned has also the dual constitution—normal immune-body + complement—as was first shown by Ehrlich and Morgenroth, naturally raised the question as to whether normal bactericidal action might not be of an analogous nature, and many observers speak as if this were established. At the present time it seems unjustifiable to take up this position. No doubt in the case of the bactericidal action on certain organisms the presence of a natural immune-body has been demonstrated, e.g. an immune-body for the anthrax bacillus in the dog's serum, which can be re-activated by rabbit's complement. On the other hand, various bacteria take up complement directly—the complement content can be practically exhausted if we use sufficient bacteria—and one can scarcely imagine that complement taken up in this way is without effect.

Attempts to obtain further insight into normal bactericidal action have chiefly been in the direction of testing specific absorbing properties, that is, treating the serum by one organism and studying any changes which may result as regards its action on other organisms.

Wright and Windsor[1] (1902) found that treating normal human serum with a small quantity of dead cholera culture removed the bactericidal action for the cholera and typhoid organisms alike, and that treatment with dead typhoid bacilli had a like result. On the other hand, organisms which are not killed by the serum, e.g. *Staphylococcus aureus*, did not, unless possibly to a very small extent, absorb the bactericidal complement. If, however, an animal were immunized against an organism, e. g. the typhoid bacillus, the increase of bactericidal action which might occur towards this bacillus did not obtain in the case of the cholera spirillum, there being apparently a specially developed immune-body to which the increase of action on the typhoid bacillus was due. Longcope[2] (1903), on the other hand, found evidence of the existence of a specific complement for the typhoid bacillus which was reduced in cases of the disease. He

[1] Wright and Windsor, *Journ. of Hyg.*, 1902, vol. ii, p. 385.
[2] Longcope, ibid, 1903, vol. iii, p. 28.

concluded that human serum contained a multiplicity of bacteriolytic complements. Buxton[1] (1905) investigated the changes produced in the serum after it had killed a certain amount of a given bacterium. His results showed that a serum which had killed the typhoid bacillus might have lost bactericidal effect on that organism, while it retained it for the paratyphoid bacillus; the converse also held good. On the other hand, the killing of cholera organisms removed the bactericidal power in the case of both the bacilli mentioned. Steinhardt[2] (1905) found that the bactericidal effect both for typhoid and dysentery bacilli was removed by treatment with dead culture of either of these organisms, and was partially restored by the addition of heated serum (natural immune-body). She ascribed the result to the presence of a common immune-body which was removed by dead culture of either organism, there being at the same time a non-specific reduction in complement. Forster[3] (1905) obtained, as regards typhoid and cholera organisms, similar results in the case of goat serum to those of Wright and Windsor in the case of human serum. He also found that a large amount of typhoid immune serum produced deviation of complement ('Neisser-Wechsberg phenomenon') for the cholera spirillum as well as for the typhoid bacillus—a result which is different from that obtained by Buxton (loc. cit.).

The subject is one of great complexity, and involves the question as to the existence of natural immune-bodies, and also that as to multiplicity and specificity of complements. At present it is not possible to bring the results of different workers into harmony and draw general conclusions. In our experiments we have used guinea-pig's serum throughout, and we may mention at the outset that we have met with considerable variations in the properties of the serum of different guinea-pigs. The results given, however, are those drawn from a large series of experiments.

[1] Buxton, *Journ. Med. Research*, 1904–5, vol. xiii, pp. 305, 431, 461.
[2] Steinhardt, ibid, 1905–6, vol. xiv, p. 161.
[3] Forster, *Lancet*, 1905, vol. ii, p. 1531.

Methods

Bactericidal action of the serum was tested by practically the same method as that used by Neisser and Wechsberg. To each of a series of small test-tubes varying quantities of fresh serum were added, usually 0·3, 0·2, 0·1, 0·05 c.c. To each tube there was then added sufficient 0·8 per cent. sodium chloride solution to make up to 0·8 c.c., and 0·1 c.c. bouillon was then added. The amount of living organism, as is shown in the tables, varied in different cases. In any case the culture was diluted so that 0·1 c.c. contained the amount to be tested. This was then added to each of the tubes. It is thus evident that each tube will contain 1 c.c., in which there is the same amount of bacteria, but varying amounts of complement. The tubes are then incubated at 37° C. for three hours. At the end of that time 0·025 c.c. is taken from each tube and added to a tube of melted agar. The agar is then plated; the plates are incubated for forty-eight hours, and the colonies counted after that time.

In the experiments regarding the modification of the bactericidal properties produced by an emulsion of one bacterium as tested on another, living cultures were at first used; for example, a minute quantity of living typhoid culture was added, the mixture was incubated for three hours, and thereafter a small quantity of *V. Metchnikovi* was added. This method was found to be attended with difficulties, as in order to procure definite results it was necessary that the first organism should be completely killed off; it was accordingly discarded in favour of another, in which the organism whose absorptive properties were to be tested was added in the dead condition, the culture having been killed at a comparatively low temperature, usually about 65° C. The dead organisms were most frequently used as a certain quantity of a twenty-four hours' bouillon culture incubated at 37° C. In testing the absorptive properties two or more series of plates were used. In each series varying quantities of complement were added, and the result aimed at was to have no colonies with the largest

amount of complement, and a large number with the smallest. A period of two hours at 37° C. was allowed in each case for absorption by the dead organisms ; the tubes of the control series containing no organisms being placed in the incubator at the same time. At the end of that time the test amount of living organisms was added to each tube, and a further incubation period of three hours at 37° C. was allowed for bactericidal action to occur.

Removal of Bactericidal Action by Homologous Bacteria

In this series of experiments we have endeavoured to find the smallest amount of dead culture of an organism which will distinctly reduce the bactericidal action of a given amount of serum for the same organism. The amount of living culture has varied, but it has been so adjusted that, with the highest amounts of normal serum, killing of the bacteria is complete, or very nearly so. The following are examples :—

Example 1.—*Vibrio Metchnikovi. Inoculation with* 0·0005 *c.c. 24-hour bouillon culture of V. Metchnikovi.*

Guinea-Pig's Serum.	*Untreated.*	*Treated with killed V. Metchnikovi (24-hour bouillon culture) for two hours at 37° C.*	
		0·001 c.c.	0·01 c.c.
	Number of Colonies.	*Number of Colonies.*	*Number of Colonies.*
0.3. c.c.	0	1	7
0.2 ,,	0	1	1200
0·1 ,,	194	1600	Many thousand.
0·05 ,,	Many thousand.	Very many thousand	∞

From this experiment it is seen that 0·001 c.c. of dead culture distinctly reduces the bactericidal effect on 0·0005 c.c. of living culture.

EXAMPLE 2.—*Bacillus dysenteriæ (Flexner). Inoculation with 0·0002 c.c. 24-hour bouillon culture of B. dysenteriæ (Flexner).*

Guinea-Pig's Serum.	*Untreated.*	*Treated with 0·001 c.c. of a killed 24-hour bouillon culture of B. dysenteriæ (Flexner) for two hours at 37° C.*
	Number of Colonies.	*Number of Colonies.*
0·2 c.c.	0	0
0·1 ,,	0	0
0·05 ,,	0	750

Here again 0·001 c.c. of dead bouillon culture has a distinct effect.

EXAMPLE 3.—*Bacillus typhosus. Inoculation with 0·00006 c.c. 24-hour bouillon culture of B. typhosus.*

Guinea-Pig's Serum.	*Untreated.*	*Treated with 0·01 c.c. killed 24-hour bouillon culture of B. typhosus for two hours at 37° C.*
	Number of Colonies.	*Number of Colonies.*
0·3 c.c.	32	35
0·2 ,,	73	121
0·1 ,,	145	Several thousand.
0·05 ,,	Several thousand.	∞

In the case of *B. typhosus* 0·01 c.c. of dead bouillon culture produces a distinct effect; in other experiments we failed to obtain any distinct reduction with 0·001 c.c. The table brings out a peculiarity which we have always found in the case of *B. typhosus* with guinea-pig's serum, namely, a want of complete killing off by the higher quantities. If we compare, for example, these results with those in the case of *V. Metchnikovi* (Example 1), we find in both instances almost the same number of colonies left with 0·1 c.c. of serum; 0·2 c.c. of serum kills off completely the *V. Metchnikovi*, but leaves seventy-three colonies of *B. typhosus*, and even 0·3 c.c. fails to kill it completely. In the strain of *B. typhosus* culture used there are apparently a small proportion of

organisms comparatively resistant to guinea-pig's complement. It seems, in view of many other experiments of similar nature, that we must suppose the existence of variations in the resisting power of organisms in the same culture (typhoid) a circumstance of considerable importance.

EXAMPLE 4.—*Bacillus enteritidis (Gaertner). Inoculation with 0·00005 c.c. 24-hour bouillon culture of B. Gaertneri.*

Guinea-Pig's Serum.	*Untreated.*	*Treated with 0·01 c.c. killed B. Gaertneri (24-hour bouillon culture) for two hours at 37° C.*
	Number of Colonies.	*Number of Colonies.*
0·2 c.c.	0	0
0·1 ,,	0	2
0·05 ,,	0	1500

Here again 0·01 c.c. has a distinct effect; results with 0·001 c.c. were negative.

It will thus be seen that in two cases distinct reduction of bactericidal action was brought about by 0·001 c.c. of dead bouillon culture, in two others by 0·01 c.c. It may be noted that there exist great variations in the proportion between the amount of organisms killed and the amount of dead culture necessary to affect the result. For example, with *V. Metchnikovi* this proportion is 1 to 2. In the case of *B. Gaertneri* it is 1 to 200. The results would on the whole go to show that the more readily an organism is killed the more easily is the result affected by previous treatment with homologous culture. This might possibly be due to the presence of a small amount of natural immune-body necessary for the process of killing, but readily absorbed by the dead culture. The subject is one, however, which involves theoretical considerations of much difficulty, and cannot at present be profitably discussed.

Removal of Bactericidal Action by Heterologous Bacteria

In this series of experiments the methods are the same as in the previous, with the single difference that the bacterium to whose absorbing properties the serum is exposed, is different from that on which the bactericidal action is subsequently tested. The following are examples :—

Example 1.—*Inoculation with* 0·0001 *c.c. 24-hour bouillon culture of B. dysenteriæ (Flexner).*

Guinea-Pig's Serum.	*Untreated.*	*Treated with killed V. choleræ (24-hour bouillon culture) for two hours at 37° C.*	
		0·01 c.c.	0·1 c.c.
	Number of Colonies.	*Number of Colonies.*	*Number of Colonies.*
0·2 c.c.	0	0	0
0·1 ,,	0	0	660
0·05 ,,	0	1	1200

Result.—Distinct reduction of bactericidal action with 0·1 c.c. of dead bouillon culture ; other experiments with 0·01 c.c. gave negative results.

Example 2.—*Inoculation with* 0·00006 *c.c. 24-hour bouillon culture of B. typhosus.*

Guinea-Pig's Serum.	*Untreated.*	*Treated with dead V. choleræ (24-hour bouillon culture) for two hours at 37° C.*	
		0·01 c.c.	0·1 c.c.
	Number of Colonies.	*Number of Colonies.*	*Number of Colonies.*
0·3 c.c.	12	10	16
0·2 ,,	16	24	190
0·1 ,,	70	150	1500
0·05 ,,	450	1500	Several thousand.

Result.—Slight reduction by 0·01 c.c. of heterologous culture, marked reduction by 0·1 c.c.

EXAMPLE 3.—*Inoculation with 0·0005 c.c. 24-hour bouillon culture of V. Metchnikovi.*

Guinea-Pig's Serum.	*Untreated.*	*Treated with killed B. typhosus (24-hour bouillon culture) for two hours at 37° C.*	
		0·01 c.c.	0·1 c.c.
	Number of Colonies.	*Number of Colonies.*	*Number of Colonies.*
0·3 c.c.	0	1	4
0·2 „	0	1	23
0·1 „	194	146	Many thousand.
0·05 „	Many thousand.	Many thousand.	∞

Result.—0·01 c.c. gives no reduction, 0·1 c.c. distinct reduction.

EXAMPLE 4.—*Inoculation with 0·00006 c.c. 24-hour bouillon culture of B. Gaertneri.*

Guinea-Pig's Serum.	*Untreated.*	*Treated with killed V. choleræ (24-hour bouillon culture) for two hours at 37° C.*	
		0·01 c.c.	0·1 c.c.
	Number of Colonies.	*Number of Colonies.*	*Number of Colonies.*
0·3 c.c.	44	420	∞
0·2 „	31	310	∞
0·1 „	26	1000	∞
0·05 „	21	Several thousand.	∞

Result.—0·01 c.c. gives distinct reduction, 0·1 c.c. great reduction. It is to be noted that the *V. choleræ* is a very active absorber of bactericidal complement, a result which Buxton also obtained.

The following table gives the general results of experiments with heterologous organisms; it shows (*a*) the dead organism whose absorbing effect is to be tested, (*b*) the smallest amount of culture which gives a distinct reduction of bactericidal action, and (*c*) the living organism on which the treated serum was tested :—

Number of Experiment.	*Dead Organism.*	*Smallest Amount of a killed 24-hour Bouillon Culture which caused distinct Absorption.*	*Living Organism.*
11	*B. typhosus*	0·1 c.c.	*V. Metchnikovi.*
29	,,	0·1 ,,	,,
35	,,	0·1 ,,	,,
43	*V. choleræ*	0·1 ,,	,,
20	*B. pestis*	0·1 ,,	,,
13	*Staphylococcus pyogenes aureus*	0·1 ,,	,,
21	*B. dysenteriæ* (Flexner)	0·01 ,,	,,
43	*V. choleræ*	0·1 ,, 0·01 c.c. (very slight)	*B. typhosus.*
34	*V. Metchnikovi*	0·1 c.c.	,,
13	*Staphylococcus pyogenes aureus*	0·01 ,,	,,
21	*V. Metchnikovi*	0·01 ,,	*B. dysenteriæ* (Flexner).
42	*V. choleræ*	0·1 ,,	,,
45	,,	0·1 ,,	,,
23	,,	0·1 ,,	*B. Gaertneri.*
42	,,	0·01 ,,	,,
46	,,	0·1 ,,	,,

We have also made a series of tests to determine what, if any, effect the amounts of dead bouillon culture which give positive results in the above table have on the hæmolytic dose of complement. In these experiments varying amounts of guinea-pig's serum were treated for two hours at 37° C. with 0·1 c.c. of dead bouillon culture of the several organisms, and the smallest amount of treated serum which gave complete lysis with the test amount of sensitized corpuscles was thereafter ascertained in the usual way, similar quantities of untreated serum being used as a control. It was found that the amount of dead culture mentioned was practically without effect on the hæmolytic dose of complement in the case of *B. dysenteriæ*, *V. choleræ*, *V. Metchnikovi*, and *B. typhosus*; in the case of *B. Gaertneri* there was a distinct increase of the hæmolytic dose, but this organism was not used as an absorbing agent in the experiments. This test for the reduction of hæmolytic

effect is a relatively more severe one than in the case of the bactericidal action ; the hæmolytic dose was about 0·007 c.c., yet the dead culture had practically no effect on this small amount, whereas in the bactericidal experiments the same amount of culture would often remove half of the bactericidal effect of 0·1 c.c. of normal serum.

The general result of these experiments is that, as a rule, 0·1 c.c. of a dead bouillon culture of heterologous organisms is requisite in order to produce a distinct fall in the bactericidal action, although in one or two instances 0·01 c.c. is sufficient. On the whole, the amount of heterologous organisms necessary to bring about the result mentioned is much greater than in the case of homologous organisms ; in fact, the results generally would appear to show that, as a rule, it is at least about ten times greater. At first sight it might appear that the explanation would depend upon the presence of an immune-body which is absorbed by the homologous organism ; whereas the heterologous organisms would act by the absorption of complement (which is present in excess in relation to the amount of immune-body) and hence a larger amount of the heterologous emulsion would be necessary. If we regard, however, the whole complement content of the serum, this explanation is not sufficient, for it is quite easy, as has just been shown, to greatly reduce the bactericidal action by means of a heterologous organism without appreciably affecting the hæmolytic value. The general results may thus be stated as follows :—

The effect of treating a serum with a given organism in the dead condition is, as a rule, first to reduce the bactericidal action for the same organism ; on increasing the amount of dead emulsion the bactericidal action on other organisms is impaired ; on still further increasing the amount of dead emulsion the hæmolytic value falls, and this may be practically exhausted, but in this case a very large amount of dead organisms must be used. It may be stated, further, that

all these effects take place more readily when the absorbing bacteria are treated with the corresponding immune-body. The fact that heterologous organisms absorb the bactericidal complement more readily than the hæmolytic complement, would point to there being a moiety of complement with greater combining affinity for bacteria in general. In fact it seems impossible, in view of the results, to escape the conclusion that this is so ; we might call this moiety ' bacteriophilic complement '.

If we now consider the fact that bactericidal action for an organism is more readily reduced by the same than by another organism, it appears that two explanations are possible. In the first place, this may be due to the existence of natural immune-bodies. The homologous organisms will absorb these immune-bodies, as well as a certain amount of complement ; hence the bactericidal action readily falls. The treatment with a heterologous organism will lead to the absorption of complement, but it will leave the immune-body for the organism on which the bactericidal action is to be tested ; it will thus reduce the bactericidal action solely by the removal of complement, but a larger amount will be necessary to bring this about. On the other hand, another explanation may hold good ; just as a moiety of the complement content is specially bacteriophilic, so in this moiety certain molecules may have a greater affinity for, and greater toxic action on, one organism as compared with another; may, for example, have a greater action on *V. Metchnikovi* than on *B. typhosus*. We have not sufficient facts at present to enable us to say which explanation is the correct one, in fact both may be valid in part ; but the fact that there exist differences in affinity of complement in relation to bactericidal and hæmolytic action respectively, certainly makes it possible that there may be variations in the affinity of complement molecules for different bacteria. Although the existence of natural immune-bodies may have been proved in certain cases, we are not justified in generalizing from this.

Reactivation of Partially Exhausted Serum

The above results clearly show that a small quantity of emulsion of a certain bacterium will reduce greatly the bactericidal action of the serum for another organism without producing a similar diminution in the hæmolytic value. A serum treated in this way may be spoken of as a partially exhausted serum. It is of course to be noted that the fall in the bactericidal action in such a serum must essentially be due to the abstraction of complement, as already explained. A serum treated with *B. typhosus* will thus still contain any natural immune-bodies it possessed for *V. Metchnikovi*, and it also contains complement (as shown by hæmolysis), yet its bactericidal action for *V. Metchnikovi* is greatly reduced. The question is—Can the complement molecules in the partially exhausted serum be rendered effective as regards bactericidal action by the addition of an artificial immune-body? In carrying out experiments on this point we have used an anti-*V. Metchnikovi* serum obtained from the rabbit, which has been inactivated at 55° C. The presence of an immune-body in this serum is shown by its greatly increasing the bactericidal action of normal serum, as well as by the greatly increased amount of hæmolytic complement taken up through its medium by a given amount of emulsion of *V. Metchnikovi* as compared with untreated emulsion. In carrying out such experiments we have used three organisms, *V. Metchnikovi*, *B. typhosus*, and *Staphylococcus aureus*, as absorbing agents, all of these being previously killed by heat. In every case reactivation occurs by the addition of the immune-body for *V. Metchnikovi*. The experiments are carried out in three series as follows :—

In series (*a*) we use the normal serum in varying amounts.

In series (*b*) we use the serum in the same amounts along with a certain amount of a dead emulsion of the organism whose absorptive power is to be tested.

And in series (*c*) we use the serum treated as in (*b*), and after time for absorption add 0·01 c.c. of anti-*V. Metchnikovi* serum.

The tubes of all three series are placed in the incubator for two hours at 37° C., and thereafter the test amount of living *V. Metchnikovi* is added, and plates are made after the usual incubation period, namely, three hours.

The three following tables give typical results of experiments of this nature :—

EXAMPLE 1.—*Partial Exhaustion by the Homologous Organism—V. Metchnikovi.*

Guinea-Pig's Serum.	*Inoculation with* 0.0005 *c.c.* 24-*hour bouillon culture of V. Metchnikovi.*		
	Untreated.	*Treated with* 0·02 *c.c. killed V. Metchnikovi* (24-*hour bouillon culture*) *for two hours at* 37° *C.*	
		+ 0	+ 0·01 *c.c. Immune-body, Rabbit versus V. Metchnikovi.*
	Number of Colonies.	*Number of Colonies.*	*Number of Colonies.*
0·3 c.c.	0	33	0
0·2 ,,	0	82	0
0·1 ,,	1	∞	0
0·05 ,,	168	∞	0
0·025 ,,	Several thousand.	∞	0

In this case the result is what one would expect on the supposition that a natural immune-body for *V. Metchnikovi* is concerned in the bactericidal action of normal serum for this organism. The previous treatment with the dead emulsion of *V. Metchnikovi* would remove the natural immune-body, hence the great fall in bactericidal effect ; whereas the addition of an artificially produced immune-body more than restores the bactericidal action by bringing complement into union with the organisms. Such an explanation will, however, not hold in the following examples, where heterologous organisms are used.

EXAMPLE 2.—*Partial Exhaustion with a Heterologous Organism—Staphylococcus aureus.*

Guinea-Pig's Serum.	*Inoculation with* 0·0005 *c.c. 24-hour bouillon culture of V. Metchnikovi.*		
	Untreated.	*Treated with* 0·1 *c.c. of suspension of dead Staphylococcus aureus for two hours at* 37° *C.*	
		+ 0	+ 0·01 *c.c. Immune-body, Rabbit versus V. Metchnikovi.*
	Number of Colonies.	*Number of Colonies.*	*Number of Colonies.*
0·3 c.c.	0	0	0
0·2 ,,	0	1	0
0·1 ,,	0	2	0
0·05 ,,	0	1350	0
0·025 ,,	270	∞	0

The result of this experiment is quite clear, and may be summed up in these words: *The treatment with dead emulsion of* Staphylococcus aureus *removes much of the bactericidal action, the addition of immune-body for* V. Metchnikovi *restores it.*

EXAMPLE 3.—*Partial Exhaustion with a Heterologous Organism—B. typhosus.*

Guinea-Pig's Serum.	*Inoculation with* 0·0005 *c.c. 24-hour bouillon culture of V. Metchnikovi.*		
	Untreated.	*Treated with* 0·1 *c.c. of suspension of dead B. typhosus for two hours at* 37° *C.*	
		+ 0	+ 0·01 *c.c. Immune-body, Rabbit versus V. Metchnikovi.*
	Number of Colonies.	*Number of Colonies.*	*Number of Colonies.*
0·3 c.c.	0	3	0
0·2 ,,	0	6	0
0·1 ,,	0	4	0
0·05 ,,	0	42	0
0·025 ,,	37	Several thousand.	0

The result exactly corresponds with the previous one, the treatment with *B. typhosus* removes much of the bactericidal action, the subsequent addition of immune-body for *V. Metchnikovi* restores it. We also obtained a similar result when the serum was partially

exhausted with *B. dysenteriæ* (Flexner); the addition of artificial immune-body restored the bactericidal action.

These results, which are of considerable importance, are at first sight somewhat puzzling. In the light of what has been stated in the previous section regarding the absorption of bactericidal complement by means of heterologous organisms, however, they seem capable of ready explanation. It has been shown that a quantity of heterologous organisms, insufficient to affect hæmolytic complement, may produce a distinct fall in the bactericidal action, and this has been explained on the supposition that there is a certain moiety of complement that has a great affinity for bacteria in general, and is most actively bactericidal. We do not mean, however, by this that the bactericidal complement is quite distinct from the hæmolytic. As a matter of fact, if we use a sufficient quantity of dead organisms, we can absorb the hæmolytic complement also. We would rather suppose that the complement molecules present all degrees of combining affinity for, and bactericidal action on, a given bacterium, and that those of greatest activity are first absorbed by bacteria in general. Thus when a certain amount of staphylococcus emulsion has acted on a given quantity of normal serum and reduced its bactericidal action for the *V. Metchnikovi*, the serum still contains any natural immune-bodies originally present for *V. Metchnikovi*, and it also contains complement. Bactericidal action, however, does not occur, the amount of the residual complement brought into combination with the organisms, directly or indirectly, being insufficient to kill them. The addition of the artificial immune-body, however, is the means of bringing so much weakly acting complement into combination that death of the organisms results. According to this view bactericidal action by the normal serum may differ from the bactericidal action produced by a partially exhausted serum with the aid of an artificial immune-body. In the

former case it is due to a few very active complement molecules being brought into combination ; in the latter, to the action of a larger number of weakly acting molecules, which combine through the medium of the artificial immune-body. In this hypothesis it is immaterial whether the normal bactericidal action takes place by the direct union of complement or by union through natural immune-bodies. The existence of complement molecules of different degrees of activity is the essential point in the explanation.

Although the subject of anti-bacterial sera produced by active immunization does not fall within the scope of the present communication, some points of interest may be referred to. In our work we have used three anti-sera, namely, anti-*V. Metchnikovi*, anti-typhoid, and anti-staphylococcus. In the first place, all these sera contain a considerable quantity of specific immune-body, as shown by the deviation or increased absorption of hæmolytic complement. The following table shows this in the case of anti-staphylococcus serum. In each tube the guinea-pig's complement is exposed to the absorbing action of a bouillon culture of *Staphylococcus aureus* ; in one series alone, in the other along with the homologous immune-body. The table shows the amount of lysis which occurs in the test amount (1 c.c. of a 5 per cent. suspension) of sensitized ox's corpuscles, added after two hours at 37° C. ; the amount of complement taken up thus becomes manifest.

Guinea-Pig's Serum.	0·1 *c.c. dead 24-hour bouillon culture of Staphylococcus aureus.*	
	+ 0	+ 0·025 *c.c. Immune-body, Rabbit versus Staphylococcus aureus.*
0·01 c.c.	Complete lysis.	0
0·025 ,,	,, ,,	Trace of lysis.
0·04 ,,	,, ,,	Very marked lysis.
0·06 ,,	,, ,,	Complete lysis.

Note.—In a control tube containing 0·01 c.c. guinea-pig's serum +

0·025 c.c. immune-body rabbit versus *Staphylococcus aureus* to which, after two hours at 37° C., the test amount of red corpuscles + immune-body was added, complete lysis resulted.

It thus seems that while 0·01 c.c. of complement treated with *Staphylococcus aureus* alone is sufficient to produce complete lysis of the test corpuscles, 0·06 c.c. is required when the complement has been treated with the *Staphylococci* + immune-body. Although the serum leads to the taking up of more complement, no bactericidal effect is brought about; in other words, even the increased amount of complement brought into combination is not lethal to the organisms. We tested the anti-staphylococcus serum in varying amounts without any bactericidal action becoming manifest. We may note that this serum when inactivated was found to contain a large amount of specific opsonin for the *Staphylococcus aureus*.

In the case of the anti-typhoid serum a marked deviation of hæmolytic complement was also produced. For example, in an experiment in every way similar to the above the hæmolytic dose of guinea-pig's serum treated with typhoid bacilli alone was 0·0075 c.c., whilst the dose when the serum was treated with typhoid bacilli + immune-body was 0·075 c.c., i.e. ten times as great. We failed, however, to produce increased bactericidal action with this serum inactivated at 55° C. in association either with guinea-pig's or rabbit's normal serum. We found, however, a Neisser-Wechsberg effect when a large amount of anti-serum (0·1 c.c.) was used, this being sufficient to practically annul the bactericidal effect of the normal serum. We also found, as Buxton did, that the fresh typhoid serum had less bactericidal effect than fresh normal serum.

In the case of the anti-*V. Metchnikovi* serum we found, in addition to the presence of an immune-body as shown by the absorption of complement, a marked bactericidal action when reactivated. In testing, however, with varying

amounts of the anti-serum 0·0001 to 0·1 c.c., we obtained no Neisser-Wechsberg effect, all these amounts greatly increasing the bactericidal effect of normal serum. This is the serum which we have employed in most of the experiments described above. It is not our purpose to discuss these phenomena at present—we mention them in order to illustrate how much the properties of an immune-serum may vary—a fact which is not sufficiently recognized. The only property which the three sera have in common is that of leading to increased absorption of complement when used in combination with the homologous organisms, i. e. the Bordet-Gengou effect. As we have shown above, by this method, which is essentially the employment of powerful complement absorbers, bactericidal complement, hæmolytic complement, and normal labile opsonin are all absorbed.

From what has already been stated it will be evident that we consider it impossible to suppose that the complement content is uniform ; that is, is made up of identical molecules. Ehrlich, as is well known, has maintained the plurality of complements, and in association with his co-workers has given examples in proof of this contention. We ourselves, in a previous section on hæmolysis (p. 77), have cited an example of this, namely, that sensitized guinea-pig's corpuscles absorb the complement for sensitized ox's corpuscles in much greater proportion than they absorb the complement for sensitized guinea-pig's corpuscles. Differences in complement molecules as regards combining affinities and certain physical properties undoubtedly exist. We have, on the other hand, given examples where the differences between complements of different animals are much greater as regards the zymotoxic group than as regards the haptophore group (p. 85). In the present series of observations the differences in the complement content which come out are quantitative rather than qualitative.

An emulsion of bacteria may absorb bactericidal complement more readily than hæmolytic complement, but if a

sufficient amount be used the latter will be taken up also. Bordet and Gengou showed that sensitized bacteria of various kinds took up hæmolytic complement, and came to the conclusion that bactericidal and hæmolytic complements are identical. Though their results are valid, their conclusion is not justified. Because various complements are taken up by powerful absorbers, it does not follow that they are all the same, and as a matter of fact weaker absorbers bring out differences. The results of the present series of experiments show that differences in combining affinity exist, but these are quantitative rather than qualitative ; though by other methods certain qualitative differences can also be established.

Conclusions

1. A distinction is to be drawn between absorption of complement by bacteria and the bactericidal effect which may follow, the latter implying a sensitiveness of the bacterium to the combined complement. Organisms may absorb complement by themselves, and to a greater extent through the medium of an artificial immune-body, without being killed.

2. Treatment of a normal serum with increasing amounts of a dead emulsion of a bacterium usually produces effects in the following order :—(*a*) diminution of the bactericidal action on the same bacterium; (*b*) diminution of the bactericidal action for other bacteria ; (*c*) diminution of hæmolytic complement.

3. Bactericidal action of normal serum for one organism can be greatly reduced by treatment with other organisms in the dead condition, without the hæmolytic effect being appreciably altered. This shows that there is a moiety of the serum which has a special affinity for bacteria in general, and is mainly concerned in bactericidal action when such occurs—it may be called *bacteriophilic complement.*

4. When the bactericidal action of normal serum for a particular organism has been greatly reduced by treatment

of the serum with heterologous organisms, the bactericidal action can be more than restored to its former value by the addition of a small quantity of the homologous immune-body (artificial anti-serum).

5. Normal bactericidal action may in certain cases differ from bactericidal action through the medium of an artificial immune-body ; in the former it may be due to the union (direct or indirect) of a few very active molecules of complement with the bacterium, in the latter to union of a larger number of more weakly acting complement molecules.

6. The fact that the bactericidal effect for an organism is usually most readily reduced by treatment with the same organism may be due to the existence of natural immune-bodies ; it may also be due, however, to variations amongst the molecules of bacteriophilic complement. This question is left undetermined.

ADDENDUM.—ON COMPLEMENTS IN GENERAL

The complement content of the serum is of prime importance in immunity, and no doubt also in the normal nutritive processes of the body. It includes a number of molecules of different kinds, or probably more correctly the molecules show an infinite number of gradations in their affinities and action, and probably also vary from time to time. We have, of course, no direct chemical method of demonstrating the existence of complement molecules. This can only be done by their effect in producing hæmolysis, bacteriolysis, &c. Complement may, however, enter into combination with various organic substances without producing any recognizable effect, the occurrence of the combination being shown by the absorption or subtraction method, which has so often been used in the foregoing investigations. This method must accordingly be employed where questions of combination are concerned. In the present state of our knowledge we cannot define complement according to the action which it produces, as there may be molecules of the

same class with activities which are not yet known. It is better to regard complement from the point of view of chemical or physical combination, and define it to be *that labile substance of normal serum which is taken up by the combination of an antigen and its anti-substance* (*immune-body*). The antigen of itself may not take up any appreciable amount of complement, e.g. in the case of hæmolysis, or it may take up a small amount as is seen in the direct absorption of the complement by bacteria and tissue cells. In this latter case the amount of absorption is, of course, much increased by the addition of the homologous immune-body. Various antigens combined with the homologous immune-bodies are thus powerful complement absorbers, and in some cases the complement content appears to be practically exhausted after such treatment. The action of complement as thus defined varies ; it may be hæmolytic, it may be bactericidal or bacteriolytic, and it may be opsonic ; in one instance an agglutinative effect has been described above.

Complement is sometimes spoken of as a ferment-like substance. There are certainly some similarities in the physical properties of ferments and complements, but if we study the mode of action, a wide difference is seen at once. So far from complement not being used up in producing its characteristic effect, we find that it enters in definite proportion into firm union with the cell on which it acts, and that in most cases there is no evidence of subsequent dissociation. The maximum amount of hæmolysis by a given amount of complement thus soon comes to an end and the process does not progress. We have also shown that the amount of complement fixed depends upon the amount of immune-body present, and that in the case of hæmolytic sera many times the amount of complement necessary for complete lysis may be fixed to the receptors of the corpuscles. In some cases each additional dose of immune-body leads to the union of additional com-

plement, and the complement fixed may be approximately proportional to the amount of immune-body present. In other cases, however, additional molecules of immune-body do not fix additional complement. In the mode of union of complement there is thus a wide difference from what obtains in the case of enzyme action.

The studies which we have carried out show that it is quite impossible to consider the complement of the serum as being made up of similar molecules. On the contrary, variations both in the combining affinities and in toxic effects are met with. In the case of the complements of the same animal, Ehrlich and Morgenroth showed differences in certain of their physical properties; for example, resistance to heat, capacity of passing through a porcelain filter, &c. We have also demonstrated a striking case where a part of the complement acts on sensitized guinea-pig's corpuscles, but not on sensitized ox's corpuscles. Perhaps, however, the most striking example is afforded in the case of absorption of complement by bacteria, the bactericidal complement as tested on one bacterium being absorbed by a great many different bacteria before the hæmolytic moiety is appreciably affected. We have, however, pointed out that so far as combining affinity is concerned the difference in this last case is rather one of degree than of quality, as if a sufficient amount of the bacteria be used the hæmolytic complement is absorbed also. It thus seems to be rather an instance of preferential absorption, the molecules with greatest affinity combining first. When powerful complement absorbers are used, on the other hand, the complements of the serum can be almost entirely removed.

Whilst therefore certain differences in the complement molecules of the same animal undoubtedly exist, these in many instances merely represent gradations in combining affinity. The haptophore group of complement does not at all show the special affinity which exists in the case of immune-body; there is a certain community in its com-

bining relationships. Even the complements of different animals may combine with the same molecules, the complement of one animal keeping out of combination the complement of another, and vice versa. It was considered for a time that the complement of each species had a relatively specific group, as the supposed anti-complement had always the maximum effect on the complement used in its production, and only slight effects on the complements of allied species. As has been shown above, however, this anti-complement action may be due simply to the deviation by the serum receptors + the anti-substances, and the existence of true anti-complements is somewhat doubtful. The result of the work on this subject, in fact, has been to emphasize the community of the combining affinities of complements of different animals. Whilst therefore we consider that the view as to the uniformity of alexine (complement) molecules of a given species of animal is not tenable, we think that Ehrlich has pushed too far the conception of special combining affinities of complement. Differences in *degree of combining affinity* have been almost completely overlooked by writers on the subject ; and when we use a powerful complement absorber, say a serum precipitate produced by the action of a precipitin on the homologous serum, the variety of complements from different animals which may be absorbed is extraordinary.

Complements of different animals, however, differ widely in their *zymotoxic* groups ; that is, in their relative toxic action. Thus it is that in order to bring about a particular effect, e.g. complete hæmolysis, a much greater amount of the complement of one animal than of another has to be brought into union, and hence a larger amount of immune-body comes to be necessary ; and in some instances a relatively large amount of complement may enter into union with a corpuscle without producing any marked lysis. This factor of relative toxicity is also seen when we use the same complement and test it on the corpuscles of

different animals, the amount which has to be taken up in order to produce lysis varying in different instances. The striking difference in the relative toxicity of guinea-pig's and rabbit's complement, when tested both on rabbit's and guinea-pig's corpuscles, is a striking example of this (p. 74). Even more striking, however, is the relative toxicity of complement when tested on different bacteria; many organisms, as already pointed out, combining with bactericidal complement, without any bactericidal action resulting. In the case of an anti-bacterial serum it is accordingly not only essential that the complement be brought into combination, or, as it is often expressed, that the complement should suit the immune-body, but also that it should have the power to produce the necessary destructive effect. We have repeatedly insisted on the importance of studying the combination of the three elements concerned, viz. receptors, immune-bodies and complements by the absorption method. The mere occurrence of hæmolysis, for example, gives no indication of the total combining affinities. Many times the amount both of immune-body and of complement necessary for lysis may be taken up, and, on the other hand, the non-occurrence of bactericidal effect, or even of hæmolysis, is no indication that complement has not entered into combination. It is only by studying the alteration in the properties of the fluid in which the particular reaction has taken place that we can judge of the substances which have been absorbed, and thus be able to understand the behaviour of the complement molecules.

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