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THE CONSTITUTION OF THE NORMAL HÆMO- LYSIN OF OX SERUM FOR GUINEA-PIG BLOOD, WITH SPECIAL REFERENCE TO FILTRATION EXPERIMENTS AND TO CON- GLUTINATION.*

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It has been found that in most instances normal hæmolysins possess the same constitution as immune lysin, *i.e.*, the lytic action depends on a thermostable body with specific combining affinity for the red cells, acting along with complement of normal serum. But in the case of the lytic property of normal ox serum for guinea-pig corpuscles, first investigated by Ehrlich and Sachs (1902⁷), certain difficulties have been met with in attempting to determine the double constitution. Thus (1) the lytic body does not possess a high degree of resistance to heat; if the serum has been heated beyond 53° to 54° C., the hæmolytic dose, as determined on the addition of complement, is much higher than when serum heated at a lower temperature is employed, but (2) more important is the observation that it is, as a rule, impossible to sensitise the corpuscles by contact with heated ox serum. The facts, as Ehrlich and Sachs record them, are as follows. Fresh horse serum in moderate doses does not contain enough antibody, along with its own complement, to cause the hæmolysis of guinea-pig blood. No hæmolysis, of course, results when the corpuscles are mixed only with inactivated ox serum. But if the corpuscles are acted on by inactivated ox serum together with fresh horse serum very marked hæmolysis is observed to occur. If, however, the guinea-pig corpuscles are treated with inactivated ox serum at 37° C. for one hour, and then the serum is removed by centrifuging, it will be found that on the addition of fresh horse serum, as complement, to the treated corpuscles

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haemolysis does not take place. The same result has been obtained by the writer, using fresh guinea-pig serum as the source of complement in place of fresh horse serum, in order to exclude the possibility of any antibody, other than that present in the ox serum, coming into action. Thus, when multiples of the hæmolytic dose of heated ox serum (53° C.) are added to guinea-pig blood suspension, and after the mixture has been allowed to stand in the ice-chest (up to eighteen hours), at 37° C. (for two and a half hours), or at room temperature (the mixture being allowed to stand overnight after preliminary incubation at 37° C. for two and a half hours, as Kolmer (1920⁸) has recommended), if the fluid is removed by the centrifuge, and the corpuscles are washed once with ice-cold saline, resuspended in saline, and fresh guinea-pig serum is then added, complete lysis does not occur subsequently at 37° C. (very slight lysis sometimes has been seen). The lysis is still present quantitatively in the fluid which has been removed. Controls with similar mixtures to which complement has been added, without removing the ox serum, undergo complete lysis. Ehrlich and Sachs concluded that their experiments showed definitely the ability of antibody in the ox serum to unite directly with complement; and they advanced the view that it was only after this antibody had combined with complement that it could react with the guinea-pig corpuscles. If this interpretation is correct, it constitutes strong evidence against the general principle of sensitisation as conceived by Bordet. Bordet and Gay (1906¹) accordingly studied the phenomenon carefully and pointed out that Ehrlich and Sachs had failed to note the significance of the agglutination of the red corpuscles which occurred in their experiments. Heated ox serum by itself has only very slight agglutinating action on guinea-pig corpuscles. Fresh horse serum agglutinates the red cells to some extent, but this occurs only slowly and when the serum is present in relatively large amounts; several hours may be required to clump the corpuscles into considerable masses. A mixture of the two sera, however, produces intense agglutination in a few minutes, and the corpuscles soon form coarse flocculi which adhere firmly to the glass. According to the analysis of Bordet and Gay, there is a moderately powerful sensitiser (normal antibody) for guinea-pig corpuscles in heated ox serum, which can be demonstrated by using a mixture of guinea-pig corpuscles, heated ox serum, and guinea-pig serum (complement). The horse serum, however, also contains a sensitiser for guinea-pig corpuscles, which is usually stronger than that present in heated ox serum; the presence of this is shown by using a mixture of guinea-pig corpuscles, unheated horse serum, and guinea-pig serum (complement). On the other hand, the complement of fresh horse serum is very deficient in hæmolytic power, for it fails to lyse ox corpuscles sensitised by specific immune body from the rabbit, as Ehrlich and Morgenroth (1901⁶) noted; the latter observers explained that horse complement does not "fit" the

rabbit sensitiser. Also, since the normal immune body of horse serum is very thermolabile, fresh serum must be used in order to demonstrate the sensitising effect. Bordet and Gay pointed out further that ox corpuscles sensitised by specific immune body from the rabbit are not agglutinated or hæmolyised either by fresh horse serum or heated ox serum; however, strong agglutination (and hæmolysis) is seen when the corpuscles are mixed with the two sera together. The heated ox serum in this case apparently possesses a peculiar substance, which has an intensifying influence upon the action of fresh horse serum. This substance was referred to by them as "bovine colloid"; afterwards it was called "conglutinin" by Bordet and Streng (1909²), and the phenomenon was termed "conglutination." It is to be noted that Muir and Browning (1906¹²) independently made the same observation in the case of ox serum and ox corpuscles sensitised with specific immune body from the rabbit. They found that on mixing red cells, immune body and ox serum, there was little or no hæmolysis at 37° C., but intense (although transient) agglutination rapidly occurred. With heated ox serum there was no agglutination. With fresh serum at 0° C. there was practically no agglutination after several hours, but if the mixture was centrifuged thereafter the corpuscles adhered into very compact masses. It was concluded, therefore, that the agglutination depended on the action of complement; but it could not be decided whether the agglutinating and the hæmolytic complement of ox serum were identical. Muir and Browning observed also in the case of the combination guinea-pig corpuscles, immune body from the rabbit and fresh ox serum, that marked agglutination occurred at 0° C., although there was no lysis.

Bordet and Gay interpreted Ehrlich and Sachs' experiment as follows. When guinea-pig corpuscles are added to a mixture of fresh horse serum and heated ox serum, they are affected by the sensitiser (antibody) of horse serum, and to a certain extent, by the sensitiser in heated ox serum. This second sensitiser is, however, superfluous, since its presence is not necessary for the phenomenon. When sensitisation is effected, the corpuscles are then in a condition to fix the horse complement. This complement, however, has only slight hæmolytic power. But once the corpuscles have become sensitised and laden with complement they are modified in their properties of molecular adhesion to such an extent that they are capable of attracting the colloidal substance in heated ox serum, which unites with them. The adhesion of this new substance produces two results; it agglutinates the blood corpuscles energetically, and also causes them to be more easily destroyed by complement. Consequently, a powerful clumping followed by hæmolysis is observed. Thus two conclusions are reached: (A) Heated ox serum treated with guinea-pig corpuscles retains its property of forming a hæmolytic mixture with horse serum, because the essential colloidal substance is not removed. Whereas the natural

immune body for guinea-pig blood, which may be removed entirely from the serum, is not required for hæmolysis. This colloidal substance is absorbed only by corpuscles treated with sensitiser and complement. (B) Treated guinea-pig corpuscles which have fixed sensitiser, but not the colloidal substance of heated ox serum, are not hæmolysed if the serum is removed and horse serum is then added. The explanation is that such corpuscles, although they are sensitised, and fix the complement in the horse serum, are not subjected to the action of the colloidal substance which has been removed along with the ox serum. Bordet and Gay's explanation appears to fit their observation that any combination of treated or untreated sera which they found to produce hæmolysis, also led to agglutination and, conversely, that where agglutination failed to appear hæmolysis did not occur. But if their view was correct one would expect, as Sachs and Bauer (1907¹⁵) pointed out, that guinea-pig corpuscles which have been placed in contact with fresh horse serum alone, and then have been washed until free from the serum, should undergo agglutination and hæmolysis on the subsequent addition of heated ox serum. Agglutination has been shown to occur in this case, but not hæmolysis. Bordet and Gay have explained the absence of lysis as due to the marked resistance to hæmolysis, which is manifested by strongly agglutinated red corpuscles. This conception, however, appears to contradict that previously formulated by the authors (see above). Moreover, Browning's experiment (1906³) definitely supports the conclusion that the normal antibody in heated ox serum acts along with the complement of fresh horse serum in bringing about hæmolysis of guinea-pig corpuscles. Thus horse serum which has been treated with guinea-pig blood at 0° C., and has thereby lost the greater part of its normal antibody, is practically as active as the native serum in causing lysis along with heated ox serum. On the other hand, horse serum treated with guinea-pig corpuscles at 37° C. has lost its power of complementing heated ox serum, since the complement is removed through the intervention of antibody in the same serum. In other words, as was pointed out by Muir and Browning (1904¹¹), a clear distinction must be drawn between (1) absence of hæmolysis due to deficiency in combining properties of complement, and (2) that due to deficiency in toxic action although combining power is active. Horse complement is highly toxic for guinea-pig corpuscles when brought into combination through the medium of the normal antibody present in ox serum, but is practically non-toxic when combined through the normal immune body of horse serum. A further experiment given by Browning afforded additional support to the view quoted above; thus horse serum which has been treated with guinea-pig blood corpuscles at 0° C., and then subsequently with a further quantity of these red cells at 37° C., still retains to a great extent its capacity for lysing guinea-pig blood along with heated ox serum; since the horse serum, having been deprived of the greater part of its

antibody by contact with the red cells at 0° C., in consequence does not lose its complement when treated with further red cells at 37° C. Bordet does not appear to have departed from his original explanation of the phenomenon.

The following work is concerned with several points of theoretical interest in connection with this problem, which have not yet been cleared up. (1) Is the antibody in ox serum incapable of sensitising guinea-pig blood in the absence of complement, as Ehrlich and Sachs stated, and (2) What is the relationship of conglutination to the other phenomena?

Methods.

0.25 c.c. of 3 per cent. suspension of well-washed guinea-pig blood was used. Fresh guinea-pig serum was employed throughout as the source of complement, suitable excess being provided by 0.1 c.c., which alone, of course, never caused lysis of the red cells. The ox serum was generally used within two or three days after the blood had been withdrawn; the M.H.D. of unheated serum is usually 0.01 c.c. for 0.25 c.c. guinea-pig blood suspension. Inactivation of the serum was effected by heating at 53° C. for half an hour. It was found, as noted by Sachs, that a higher temperature, *e.g.*, 55° C., usually causes great weakening of the antibody content of the serum; but occasionally serum heated at 53° C. was slightly lytic by itself in large amounts, *e.g.*, 0.3 c.c. In the presence of excess of complement the M.H.D. of serum which has been heated at 53° C. ranges from 0.03 to 0.05 c.c. Occasionally serum heated at 53° C. appears to be very weak in antibody, but this may be due to the anti-complementary action of the heated serum masking the presence of the hæmolysin.

THE COMBINING AFFINITY OF THE NORMAL ANTIBODY IN OX SERUM FOR GUINEA-PIG BLOOD.

(1) *With Heated Ox Serum.*—As mentioned above, heated ox serum lacks the power of sensitising guinea-pig blood. Thus 1 to 4 M.H.D. of ox serum 53° C. were added to the blood suspension, and after contact at various temperatures and for varying periods, as above detailed, the fluid was removed quickly by the centrifuge, and the sediment was washed with an amount of ice-cold saline, equal to one or two volumes of the original suspension; the sediment was then resuspended in saline and complement was added, but lysis did not occur in this mixture when it was allowed to stand one and a half hours at 37° C. (Neufeld's (1918¹⁴) experiments with an exceptionally lytic specimen of horse serum need not be discussed.) It is important to note at the outset that there is a difference in the method of experiment pursued by Ehrlich and Sachs, and by Bordet with Gay and with Streng. The treatment of heated ox serum with guinea-pig corpuscles was carried out by Ehrlich and Sachs in a series of tubes, containing varying amounts of serum considerably diluted with salt solution; on the other hand, Bordet and Streng treated one volume of heated ox serum, added to one volume of saline, with one volume of washed guinea-pig blood. Both authors' methods have been re-examined, and it was confirmed

that antibody was removed to a certain extent by red corpuscles according to Bordet's procedure, since the treated serum was deficient in lytic power as compared with the untreated serum when added to guinea-pig blood suspension and complement. However, corpuscles which have been treated in this way and then freed from serum do not lyse at 37° C. when complement is added.

The affinity of the antibody for heated guinea-pig stromata was next investigated. The preparation of stromata was carried out by the method originally described by Sachs.

Ten per cent. suspension of well-washed guinea-pig corpuscles was heated in the water bath at 55° to 56° C. for three to four hours as recommended by Kosakai (1920⁹), then washed with saline till the supernatant fluid became colourless and finally resuspended in the original volume. 1 to 4 M.H.D. of heated ox serum (as reckoned for 0.25 c.c. 3 per cent. blood suspension) were added to 0.25 c.c. 10 per cent. suspension of guinea-pig heated stromata. The fluid was separated by centrifuging after the mixtures had stood for two hours at 37° C. or three hours at 0° C., and was added to the sediment of 0.25 c.c. guinea-pig blood suspension, 0.1 c.c. guinea-pig complement was then added and the tubes were kept at 37° C. for one and a half hours. As controls, similar amounts of heated ox serum were added to salt solution without stromata. The fluid from the first tube (containing 1 M.H.D. of serum) caused distinct lysis, while its control lysed completely.

Thus very little of the antibody had been bound by the stromata.

Ehrlich and Sachs suggested that the antibody attaches itself to guinea-pig corpuscles in the presence of complement. But the addition of a small amount of guinea-pig complement, which sufficed to cause only a trace of lysis of the test corpuscles at 37° C., did not produce any notable increase in the amount of antibody which was fixed on contact with heated ox serum.

Example. Series A contained 0.25 c.c. guinea-pig blood suspension, and series B, as control, 0.25 c.c. of saline in each tube; 2.5 M.H.D. of heated ox serum (along with 0.1 c.c. complement, M.H.D. = 0.04 c.c.), and ascending amounts of fresh guinea-pig serum (0.01 to 0.05 c.c.) were added to the tubes of both series; the mixtures were allowed to stand in contact for one and a half hours at 37° C.; the initial lysis in the tubes of guinea-pig blood suspension was as follows:—

With 0.01 c.c., complement, none			
„	0.02 c.c.,	„	trace
„	0.03 c.c.,	„	distinct
„	0.04 c.c.,	„	marked
„	0.05 c.c.,	„	very marked

Then the supernatant fluids were removed by centrifuging and were added to 0.5 c.c. 3 per cent. suspension of ox corpuscles sensitised with five doses of homologous immune serum from rabbit. The treated sediments were washed once with ice-cold saline, each suspended in the original amount of saline, and 0.1 c.c. complement added. The mixtures were then kept for one and a half hours at 37° C. No further lysis occurred in the mixtures. The supernatant fluid from the first tube caused very marked lysis in series A, and almost complete lysis in series B; the second tubes were complete in both. (M.H.D. of

complement for 0.5 c.c. sensitised ox corpuscles = 0.0025 c.c.) Thus slight inhibition or deterioration of complement had occurred, as shown by series B, while series A proves that the red corpuscles had caused a certain, though small amount of complement to be fixed in addition.

Sachs and Bauer¹⁵ stated that hæmolysis was actually more rapid when horse serum and inactive ox serum were added to guinea-pig blood after the two sera had stood in contact for a time at 37° C., than when sera and corpuscles were mixed immediately before incubating. A repetition of this experiment, using guinea-pig complement and ox serum 53° C. showed that hæmolysis was always less marked and occurred more slowly when the mixture of guinea-pig complement and heated ox serum was allowed to stand for one hour at 37° C. before adding corpuscles. To avoid fallacy in control series, the reagents were incubated similarly but without mixing. The results correspond with those of Bordet and Streng.

(2) *With Fresh Serum*.—As fresh ox serum has strong hæmolytic action for guinea-pig blood at 37° C., the antibody in fresh ox serum was removed by contact with heated guinea-pig stromata. The addition of complement to the supernatant fluid does not then restore its hæmolytic action; ox stromata, by comparison, are practically devoid of such effect.

TABLE I.

No. of M.H.D. of Fresh Ox Serum.	Lysis of Guinea-pig Blood by Fresh Ox Serum previously treated with						Control.		
	Guinea-pig Stromata.			Ox Stromata.					
	A1.	A2. (A1+0.1 c.c. Comple- ment.)	A3. (A1+0.025 c.c. Ox Serum 53°C.)	B1.	B2. (B1+0.1 c.c. Comple- ment.)	B3. (B1+0.025 c.c. Ox Serum 53°C.)	C1.	C2. (C1+0.1 c.c. Comple- ment.)	C3. (C1+0.025 c.c. Ox Serum 53°C.)
1	0	ft _r	ft _r	d	m	d	vm	c	m
2	ft _r	d	vm	c	c	ac	c	c	c
3	tr	m	jc	c	c	c	c	c	c
4	d	c	c	c	c	c	c	c	c

In this and the following tables the degrees of lysis are recorded thus :—

c = complete lysis.	d = distinct lysis.
jc = just complete lysis.	tr = trace of lysis.
ac = almost complete lysis.	ft = faint trace of lysis.
vm = very marked lysis.	0 = no lysis.
m = marked lysis.	

Example. Three series of tubes were prepared, each consisting of three sets. Each tube in series A contained 0.25 c.c. 10 per cent. guinea-pig stromata, in series B 0.25 c.c. 10 per cent. ox stromata, in series C 0.25 c.c. saline. 1 to 4 M.H.D. of fresh ox serum were added to the tubes of each series (M.H.D. = 0.01 c.c.). The mixtures were allowed to stand for two hours at 37° C., and the supernatant fluid from each tube was removed by centrifuging and added to the sediment of 0.25 c.c. 3 per cent. guinea-pig corpuscles without any further

addition in sets A1, B1, C1, along with 0.1 c.c. guinea-pig complement in each tube of sets A2, B2, C2, and with a half M.H.D. of heated ox serum (M.H.D. = 0.05 c.c.) in each tube of sets A3, B3, C3. Then the mixtures were kept at 37° C. for one and a half hours. The results are shown in Table I.

A similar experiment in which the serum was treated with stromata in the ice-chest, proved that antibody was less actively fixed than at 37° C. Further experiments on similar lines showed that heated guinea-pig stromata could not deprive the serum of its normal antibody for rabbit corpuscles, but that a portion of the antibody for human corpuscles was removed thereby. Thus the normal immune body in ox serum possesses a certain degree of specificity. This specific binding property in the stromata was lost when the stromata had been treated with osmic acid as has been frequently shown (see Kosakai⁹).

Since the above experiment has shown that a portion of the antibody in fresh ox serum is bound by guinea-pig stromata, it might be expected that guinea-pig corpuscles could be sensitised in the ice-chest when they were allowed to remain in contact with fresh ox serum. But experiments confirmed the original statement that this was not the case.

Example. M.H.D. of fresh ox serum for 0.25 c.c. 3 per cent. guinea-pig blood suspension = 0.015 c.c.; 1 to 4 M.H.D. of fresh ox serum were added to 0.25 c.c. guinea-pig blood; after the mixtures had stood for two hours in chopped ice, the serum (F) was removed by centrifuging, and added to the sediment of 0.25 c.c. guinea-pig blood suspension. The treated corpuscles (S) (which adhered into very compact masses on centrifuging) were washed once with 1.0 c.c. ice-cold saline and resuspended in 0.25 c.c. saline, and 0.1 c.c. complement was added. After incubating the mixtures for one and a half hours at 37° C., the results were as shown in Table II. Controls were incubated without separating the serum.

TABLE II.

No. of M.H.D.	1.	2.	3.	4.
S	0	0	ftc	ftc
F	d	ac	c	c
Controls	all c.			

(3) *With Filtered Serum.*—It has been confirmed by many workers that a number of organic and inorganic substances can absorb the complement of a serum. Thus Ehrlich and Morgenroth (1900⁵) found that the complement of goat serum which acted with the natural lysin for rabbit corpuscles was retained by filtering, while that acting on guinea-pig corpuscles passed through. They also stated that the normal immune body for rabbit corpuscles passed through the filter. Muir and Browning (1909¹³) investigated particularly the filtration of

hæmolytic complement in guinea-pig serum, and they found that practically the whole of the complement was retained on filtering through a Berkefeld filter. The property possessed by a filter of stopping complement, however, was soon lost in the process of filtration. They also examined the behaviour on filtration of a mixture of complement (fresh guinea-pig serum) and immune body (serum of a rabbit treated with ox corpuscles, deprived of complement by heating at 55° C.) after the mixture had been allowed to stand for about an hour at 37° C. prior to filtration; they found that the immune body passed through the filter unchanged in amount, the complement, on the other hand, underwent a marked reduction, just as in the previous experiments. Kolmer (1920⁸) has stated that human serum after filtration through a Berkefeld filter lost its natural hæmolytic power for sheep and guinea-pig corpuscles, and that the addition of fresh guinea-pig serum to the filtrate restored its hæmolytic action. Schmidt (1914¹⁰) has also made extensive investigations on the filtration of hæmolytic complement of fresh guinea-pig serum; he failed to restore the hæmolytic power of filtrate by adding to it either "end-piece" or "middle-piece" of complement. This was in agreement with the conclusion of Browning and Mackie (1912⁴), who had shown that fresh guinea-pig serum filtered through a Berkefeld filter could not be reactivated as regards its hæmolytic power for ox corpuscles along with immune body, or for cobra venom by adding "end-piece" or "middle-piece" of complement. Thus it is now generally agreed that hæmolytic complement is retained on filtering serum through a Berkefeld filter, while normal antibody, as well as immune body, passes through.

The writer has also employed the method of filtration to inactivate fresh ox serum.

Small (5·7 × 1·5 cm.) and large (11·7 × 2·6 cm.) Berkefeld candles were used. The latter were preferable when a considerable amount of filtrate was required. As stated by many workers, Berkefeld filters have often a distinctly alkaline reaction, therefore in all cases before using a filter, water was passed through it by means of a vacuum pump till the filtrate showed no trace of alkalinity when tested with phenolsulphonephthalein. The filters were then dried at 100° C. for twenty-four hours. Great difficulty was met with in the attempt to remove completely the alkalinity from filters by washing. Thus when filters were employed after a preliminary washing as described above, it was found that, on passing further quantities of distilled water through the dried filter by gravitation, the first few cubic centimetres, according to the size of the filter, might be neutral; but later portions were usually distinctly alkaline. If water was allowed to pass through such a candle till the filtrate again ceased to be alkaline and it was dried, the reaction of a further filtrate would again give an alkaline reaction. Small filters have been successfully freed from alkalinity by washing and drying them two or three times, but it has not been possible to render the large filters entirely free from alkalinity by this means.

Since the permeability for complement varies according to the period of filtration, the filtrate was taken in separate fractions of

1 to 3 c.c. according to the size of the filters. As it was impossible to separate the filtrate in fractions without loss when the vacuum pump was employed, the filtrate was allowed to gravitate through the candle. The successive portions of filtrate were tested for (1) lytic power (*i.e.*, the presence of both antibody and complement); (2) antibody (by adding excess of guinea-pig complement to the filtrate); (3) complement (by adding 1 M.H.D. of ox serum 53° C. to the filtrate); (4) the occurrence of agglutination (presence of congenitinin); in addition to testing the agglutinating power of filtrates by themselves, they were also tested along with pig serum which, according to Leschly (1916¹⁰), produces agglutination in the presence of small amounts of the congenitinin of ox serum. In a series of twenty-three different filtration experiments, it was found that these various properties were on the average distributed among the fractions of filtrates as shown in Table III.

TABLE III.

Portion of Filtrate.	Lytic Power for Guinea-pig Blood.			Conglutination for Guinea-pig and Sensitised Ox Blood.	
	Alone.	On the addition of Ox Serum 53° C.	On the addition of Guinea-pig Complement.	Alone.	On the addition of Pig Complement.
1	0	0	0	0	0
2	0	0	+	0	0
3	0	0	+	+	+
4 }	Weak +	Weak +	+	+	+
5 }			+	+	+
6	+	+	+	+	+

+ Indicates the occurrence of lysis or agglutination in the respective columns.

Thus the first portion lacked normal immune body, complement, and congenitinin, the second portion contained normal antibody only, the third portion contained immune body and congenitinin, while all of these components were present in the later portions. But such sharp separation of the properties was not always obtained.

Example. (M.H.D. of fresh ox serum for 0.25 c.c. of 3 per cent. washed guinea-pig blood suspension = 0.01 c.c.). Serum was filtered through a washed Berkefeld candle (11.7 × 2.6 cm.) by gravitation (serum being maintained at the level of the top of the filter candle); each portion of 2.5 c.c. was kept separate; (a) 0.15 c.c. of each portion was tested for hæmolytic power by itself (as above). With portions 1 to 3 no lysis resulted; with portions 4 and 5 a trace; with portion 6 almost complete lysis; (b) in the presence of guinea-pig complement (0.1 c.c.), 0.06 c.c. of the first portion caused no lysis, with 0.03 c.c. of the second portion there was almost complete lysis, and 0.03 c.c. of the succeeding portions caused complete lysis. Conglutination occurred in the third and the succeeding portions (but not in the first and second) when the mixture of 0.05 to 0.15 c.c. of filtrate, and 0.25 c.c. of 3 per cent. guinea-pig corpuscles were kept in contact at 37° C. Ox corpuscles sensitised with four doses of homologous immune serum from the rabbit behaved similarly. Agglutination

developed in the mixtures after standing more than an hour at 37° C., whereas Muir and Browning and Bordet stated that agglutination (conglutination) occurred within a few minutes in the mixture of sensitised ox corpuscles and fresh ox serum at 37° C. If fresh pig serum which had been treated in the usual way with an equal volume of the respective washed blood sediment for one hour at 0° C., was added to the mixtures of filtrate along with guinea-pig corpuscles or sensitised ox corpuscles in amounts of 0·005 to 0·002 c.c. intense conglutination occurred rapidly (treated pig serum by itself in the largest amount used had no obvious effect upon guinea-pig corpuscles or sensitised ox corpuscles). It is noteworthy, however, that the addition of pig serum did not cause agglutination in the presence of the second portion of filtrate, although the latter contained a large amount of the natural lytic antibody. Accordingly, the second portion of filtrate was selected with a view to investigating the behaviour of the lytic antibody (along with 0·1 c.c. guinea-pig complement M.H.D. of this portion = 0·03 c.c.). It was found that when an amount of the filtrate containing about two M.H.D. or more of antibody was left in contact with guinea-pig corpuscles at 37° C. for two and a half hours, and three hours further at room temperature, the red cells became sensitised. Thus when the treated red cells (S) were freed from fluid and subsequently washed once with ice-cold saline and resuspended in the original amount of saline, with the addition of guinea-pig complement, lysis occurred in the incubator (the details of the experiment are similar to those given on p. 160). The fluid (F) was also tested for its content in antibody by adding it to the sediment of guinea-pig blood suspension along with complement. In the control series complement was added to the mixture of red corpuscles and filtered serum which had not been separated by centrifuging. The results were as shown in Table IV.

TABLE IV.

No. of M.H.D. (filtered Ox Serum, Portion 2).	Lysis resulting after adding Complement to		
	S.	F.	Control
1	tr	ac	} all c.
2	ac	ac	
3	c	c	
4	c	c	

S = sediment treated with filtered ox serum, and then resuspended after washing.

F = fluid obtained on centrifuging S on the first occasion.

Hence it was shown that filtered ox serum, which is devoid of agglutinating action, possesses active sensitising power for guinea-pig blood, so that corpuscles which have been treated with 2 M.H.D. of filtered ox serum bind practically one dose. Sensitisation by filtrate was less effective at room temperature or in the ice-chest than at 37° C.

Effect of Heating Filtrates.—The antibody in filtrate lost its affinity for guinea-pig red corpuscles on heating at 53° C. for half an hour, but it still caused lysis in the presence of complement although more

of the heated than of the unheated filtrate was required to effect complete lysis.

Example. M.H.D. of fresh ox serum = 0.015 c.c.; this serum was filtered as described previously (along with guinea-pig complement M.H.D. = 0.025 c.c.); after heating at 53° C. for thirty minutes M.H.D. of filtrate = 0.04 c.c. 1 to 4 M.H.D. of filtrate before and after heating were added to 0.25 c.c. 3 per cent. red corpuscle suspension and allowed to stand at 37° C. for two hours, and for three hours further at room temperature. Then the mixtures were centrifuged, both fluid and sediment being preserved; the treated sediments (S) after washing with 0.5 c.c. ice-cold saline were resuspended in the original amount of saline; the fluid was added to the sediment of 0.25 c.c. red cell suspension; finally, guinea-pig complement was added, and the mixtures were incubated as usual. The results are shown in Table V.

TABLE V.

No. of M.H.D.	Filtrate.		Filtrate, 53° C.		Control.
	S	F.	S.	F.	
1	0	c	0	c	} all c.
2	tr	c	0	c	
3	c	c	fttr	c	
4	c	c	fttr	c	

In the control series complement was added to the mixture of red corpuscles and filtrate heated at 53° C., which had not been separated by centrifuging.

Thus the results with the filtrate heated at 53° C. correspond with those obtained with unfiltered heated serum.

Since serum, both heated unfiltered and when heated after filtering, lacks sensitising power, the question arises whether sensitisation is facilitated by some non-specific body, which, being thermolabile, may be of the nature of complement. It has been seen already that sublytic doses of guinea-pig complement did not notably increase the amount of antibody absorbed by guinea-corpuscles in contact with heated ox serum. The point was investigated further in the case of the filtrate by treating the latter with agents known to absorb complement, *e.g.*, emulsion of a culture of *staphylococcus aureus* (excess of heated ox stromata sensitised with homologous immune body from the rabbit was employed, but was less efficient as a means of absorbing the complement). The result was that the antibody in the treated filtrate still retained power of combining with the corpuscles. Thus the evidence so far does not support the view that combination is due to the mediation of a complement-like body.

Example. M.H.D. of fresh ox serum for 0.25 c.c. 3 per cent. guinea-pig blood = 0.01 c.c. The filtration of the serum was carried out as already described. Portions 3 and 4 contained antibody but no agglutinin, so they were mixed and used for the experiment (along with guinea-pig complement M.H.D.

= 0.03 c.c.). 1 to 4 M.H.D. were added to tubes containing emulsion of agar culture of *staphylococcus aureus* (which had been previously heated at 70° C. for thirty minutes, and was able to absorb the complement in 0.06 c.c. of fresh ox serum, as tested with 0.25 c.c. guinea-pig blood, *i.e.*, 6 M.H.D.); the mixtures were allowed to stand one hour at 37° C. (slight agglutination of the cocci was found in the tube containing 4 M.H.D. of serum). The treated serum was separated and tested in the usual manner for sensitising power and content in antibody (as on p. 164). The results are shown in Table VI.

TABLE VI.

No. of M.H.D.	Sensitising Power of Filtrate					
	treated with Emulsion of <i>staphylococcus aureus</i> Culture.			untreated.		
	S.	F.	Control.	S.	F.	Control.
1	ft	tr	tr	ft	m	ac
2	tr	m	ac	d	ac	c
3	vm	ac	c	ac	c	c
4	ac	c	c	c	c	c

In the control series complement was added to the mixture of red corpuscles and filtrate (treated and untreated respectively), which had not been separated by centrifuging.

Thus treating with an emulsion of *staphylococcus aureus* had little effect on the sensitising power of the filtrate.

The Effect of filtering Heated Serum.—It has been found that the M.H.D. of heated ox serum was decreased when the serum was filtered through a Berkefeld filter; *e.g.*, along with 0.1 c.c. guinea-pig complement, 0.05 c.c. of ox serum 53° C. caused only marked lysis of the test corpuscles, while 0.04 c.c. of the serum after filtering completely lysed the corpuscles. This suggests that some anti-complementary substance in heated ox serum is removed by filtration.

THE NATURE OF CONGLUTINATION AND ITS RELATION TO HÆMOLYSIS.

As described already, fresh ox serum has the property of causing marked agglutination when added to certain species of red corpuscles, *e.g.*, from the ox, along with specific immune body. Heating at 56° C. deprives the serum of this property, but it may be restored when, in addition to the heated ox serum, fresh serum (complement) is present. It has been found that *pig* serum is especially suitable as a source of complement for this purpose, and agglutination is produced by adding amounts of pig serum which are too small to cause a notable amount of hæmolysis (Leschly (1916¹⁰)). Thus, to 0.25 c.c. of 3 per cent. ox corpuscles, sensitised with four doses of immune body from the rabbit, were added ox serum 53° C. and pig serum previously treated with an

equal volume of washed ox corpuscles for one hour at 0° C. The resulting agglutination after the mixtures were allowed to stand for one hour at 37° C. is illustrated in Table VII.

TABLE VII.

Ox Serum 53° C. in c.c.	0.25 c.c. of 3 per cent. Sensitised Ox Corpuscles + treated Pig Serum in c.c.			
	0.05.	0.01.	0.002.	0.001.
0.2 { A : L :	+++ --	+++ --	± --	-- --
0.05 { A : L :	+++ m	+++ --	+++ --	+++ --
0.01 { A : L :	-- c	+++ --	+++ --	+ --
0 { A : L :	-- c	-- --	-- --	-- --

A = agglutination. L = lysis.

(In this and the following tables the degree of agglutination is indicated by the number of + signs, from 3 downwards.)

No agglutination occurred in the mixture of sensitised ox corpuscles and ox serum 53° C. in the amounts used in the main test.

According to Bordet and Gay, the conglutinin in ox serum, which they regarded as distinct from antibody or complement, may be essential for the production of hæmolysis, *e.g.*, in the mixture of guinea-pig corpuscles, fresh horse serum, and heated ox serum. Hence the ox serum is regarded as acting only in virtue of its containing conglutinin (bovine colloid). Conclusive experimental evidence in support of this view is difficult to obtain, because there are natural antibodies for guinea-pig corpuscles both in the serum of horse and ox; and further, because the antibody in ox serum can not be satisfactorily removed, *e.g.*, by contact with a large amount of guinea-pig blood. As has been seen, when fresh ox serum is passed through a Berkefeld filter, it is found that the earliest portion contains neither antibody nor complement; the next portions contain antibody but no hæmolytic complement, and the earlier of these do not cause agglutination, either alone or on the addition of fresh pig complement, hence they are devoid of conglutinin. Subsequent portions contain antibody and cause agglutination, although they are not lytic by themselves in relatively large amounts. The last portions are lytic. The fact that certain parts of the filtrate contain antibody but no agglutinin, affords definite evidence that the agglutinating action (due to so-called conglutinin) is not essential for hæmolysis of guinea-pig corpuscles by complement and ox serum.

The further question remains as to what is the nature of conglutinin.

Both Muir and Browning, and also Bordet and Gay pointed out originally that complement was required for the agglutinating action. In the present experiments it has been seen that agglutination is produced by certain portions of filtrate which are practically devoid of hæmolytic complement although containing antibody. In view of the fact that minute amounts of fresh pig serum (Leschly) bring about conglutination, it is possible that the agglutinating portion of the filtrate may act in virtue of containing a trace of complement. But it is noteworthy that the earliest portion of filtrate containing antibody does not cause agglutination even when pig serum is added. Thus, in confirmation of the view of Bordet and his co-workers, it would appear that some distinctive property of ox serum is essential for the phenomenon. The attempt has been made to treat agglutinating filtrate with complement absorbing agents, *e.g.*, killed *staphylococcus aureus* culture, in order to determine whether the agglutinating property is removed.

Example. Filtration of fresh ox serum (M.H.D. = 0·01 c.c.) was carried out as described above, portions each of 2·5 c.c. were kept separate. Lysis was produced by the seventh and succeeding portions when 0·15 c.c. of each filtrate, and 0·25 c.c. of 3 per cent. guinea-pig blood suspension were mixed and allowed to stand two hours at 37° C.; marked agglutination was produced by portions 5 to 8, with and without the addition of treated pig complement (0·002 c.c.); agglutination of sensitised ox corpuscles was caused by portions 8 and 9, and on adding treated pig complement agglutination was produced also by portion 7, but was very weak compared with that of guinea-pig corpuscles. Therefore portions 5 and 6 were selected; these were mixed. The agglutinating action of the mixed serum (= filtrate) was tested after treatment with emulsion of a culture of *staphylococcus aureus*; 0·05, 0·1, 0·15, and 0·2 c.c. of the serum were added to tubes containing each 0·25 c.c. of *staphylococcus* emulsion (see p. 164), slight agglutination of the cocci was found in the third and fourth tubes when the mixtures were allowed to stand for one hour at 37° C., then the serum from each tube was removed by centrifuging, and added to the sediment of 0·25 c.c. guinea-pig blood suspension, after which the mixtures were incubated for one and a half hours at 37° C. The results are shown in Table VIII.

Accordingly the agglutinating action of filtered serum was removed by treatment with an agent which was able to absorb complement and was restored by the addition of a minute amount of fresh pig serum.

Although the agglutinating property of filtrate is extremely thermostable, being destroyed by heating for thirty minutes at 45° to 48° C., the sensitising power is still present to a great extent after such treatment.

Example. The filtration of fresh ox serum (M.H.D. = 0·01 c.c.) was carried out as mentioned before. 0·15 c.c. of portions 1 to 6 caused no lysis of 0·25 c.c. of guinea-pig blood suspension in two hours at 37° C., agglutination resulted with portions 4 to 7. These lost their agglutinating action on heating for thirty minutes at 45° C. and 48° C. The corpuscles which had been in contact with these heated sera were washed once with 1·0 c.c. of ice-cold saline then resuspended in 0·25 c.c. saline and complement was added. Complete lysis resulted at 37° C., thus showing that the filtrates heated at these temperatures retained to a great extent the power of sensitising the corpuscles.

TABLE VIII.

Amounts of Filtrate in c.c.	Treated Filtrate.		Treated Filtrate + 0.002 c.c. Pig Serum.		Controls.			
					Untreated Filtrate.		Untreated Filtrate + 0.002 c.c. Pig Serum.	
	A.	L.	A.	L.	A.	L.	A.	L.
0.1	-	-	-	-	-	-	+	ft
0.15	-	-	++	ft	++	-	+++	tr
0.2	-	-	++	ft	++	-	+++	tr

A = agglutination. L = lysis.

Neither agglutination nor lysis occurred in the mixture of guinea-pig red cells and treated fresh pig serum (0.002 c.c.).

The mixed filtrate was also deprived of its agglutinating action by heating for thirty minutes at 45° C., and it was restored to a certain extent by adding pig complement (0.002 c.c.); but a trace of lysis also occurred in this mixture.

On the other hand, sera which had been kept for some hours after filtration showed a great decrease both in agglutinating and sensitising power, although they were still fairly active in producing hæmolysis in the presence of complement. This lability of filtrates, and also the fact that different specimens of serum exhibit varying behaviour as regards the distribution of sensitising and agglutinating power in successive portions of filtrate, have made it difficult to formulate general results. Alteration in hydrogen-ion concentration due to traces of alkali derived from the filter does not appear to be responsible for the labile characters, since the addition of a suitable amount of diluted hydrochloric acid, so as to make the filtrate of the same reaction as fresh unfiltered serum, did not prevent the usual deterioration.

Example. Fresh serum (M.H.D. = 0.01 c.c.) was filtered through a washed Berkefeld filter. Portions each of 2.5 c.c. were kept separate. 0.2 c.c. of each portion was tested for hæmolytic power by itself for 0.25 c.c. of guinea-pig blood suspension; with portions 1 to 5 no lysis resulted; with portions 6 to 7 a trace, with portion 8 marked, and with portion 9 complete lysis occurred. In the presence of guinea-pig complement (0.1 c.c.), 0.06 c.c. of the first and second portions caused no lysis, with 0.04 c.c. of the third portion there was a faint trace of lysis, and 0.03 c.c. of fourth portion caused almost complete lysis; 0.03 c.c. of succeeding portions lysed completely. When 0.2 c.c. of each filtrate was mixed with guinea-pig corpuscles and stood for two hours at 37° C., strong agglutination was found in the mixture of portions 6, 7, and 8; no agglutination, however, occurred with portions 1 to 5, even when treated pig complement (0.002 c.c.) was added; but the addition of this caused lysis with the fourth and succeeding portions, while the same amount of pig serum by itself had no obvious effect upon guinea-pig corpuscles. Agglutination of sensitised ox corpuscles, however, was weak, and it was only observed in the mixture of 0.25 c.c. corpuscle suspension and 0.2 c.c. of filtrate of portions 9 and 10 after

standing for two hours at 37° C. On the addition of treated pig complement (0·005 c.c.) the agglutination was increased in portions 8 and 9, but a trace of lysis also occurred, although the pig complement by itself was without effect on sensitised ox corpuscles. As agglutination of guinea-pig corpuscles was not caused by portions 4 and 5 of the filtrate, these were mixed (M.H.D. along with guinea-pig complement = 0·03 c.c.) and used for further investigation; as the reaction of the mixture was slightly more alkaline than that of unfiltered serum, it was brought to the same reaction, as tested, with phenolsulphonephthalein, by adding dilute hydrochloric acid. Then the sensitising power was tested immediately in the case of the unadjusted filtrate and after eighteen hours, both the adjusted and unadjusted filtrates being tested on the latter occasion. The method of the test was the same as before (see p. 164). The results are shown in Table IX.

TABLE IX.

Amount of Serum in c.c.	Sensitising Power of Filtrate.								
	Immediately.			After eighteen hours.					
	Unadjusted Filtrate.			Unadjusted Filtrate.			Adjusted Filtrate.		
	S.	F.	Control.	S.	F.	Control.	S.	F.	Control.
0·03	ftr	vm	} all c	ftr	tr	d	ftr	tr	d
0·06	vm	c		ftr	vm	ac	ftr	d	vm
0·1	c	c		d	ac	c	d	ac	c
0·2	c	c		m	c	c	m	c	c

Portion 6 was dealt with similarly in order to test the effect of reaction on deterioration of agglutinin. 0·25 c.c. of guinea-pig blood suspension, and 0·2 c.c. of filtrate were used; the method was the same as already described. The results are shown in Table X.

TABLE X.

	Agglutinating Power of Filtrate.				
	Immediately.	After eighteen hours.			
	Unadjusted Filtrate.	Unadjusted Filtrate.	Unadjusted Filtrate + treated Pig Complement, 0·002 c.c.	Adjusted Filtrate.	Adjusted Filtrate + treated Pig Complement, 0·002 c.c.
Agglutination	+++	--	±	—	—
Lysis	ftr	--	m	—	m

No lysis was found in the mixture of 0·25 c.c. guinea-pig blood suspension and 0·002 c.c. of treated pig complement.

SUMMARY.

(1) Hitherto difficulties have attended attempts to demonstrate that the natural hæmolysin of ox serum for guinea-pig red blood cells possesses the usual dual constitution of immune body and complement. Thus the lytic body is relatively thermolabile, since ox serum which has been heated beyond 53° to 54° C. can usually be "reactivated" to only a slight extent by the addition of fresh serum (complement). Also, it is as a rule impossible to sensitise the corpuscles by contact at various temperatures with ox serum which has been heated to 53° C., or with fresh ox serum at 0° C. (Ehrlich and Sachs). The antibody in the heated serum is not entirely devoid of affinity for the red cells, as is shown by the fact that when the heated serum is treated with excess of red corpuscles at 37° C. and the fluid is then separated, it is found to have undergone marked diminution in lytic power, as tested in the presence of complement (Bordet and Gay). The present work has shown, however, that fresh ox serum which has been inactivated by filtration through a Berkefeld filter is capable of sensitising guinea-pig red cells, these cells after being washed undergoing lysis on the addition of complement. Hence it appears that the lytic effect of ox serum on guinea-pig corpuscles is due to the co-operation of complement with an antibody. Thus it has been found that the peculiarities of the action of the normal hæmolysin of ox serum for guinea-pig corpuscles depend on (a) the lability of the antibody, (b) its feeble affinity for the corpuscles, and (c) the interfering action of certain substances in the serum, these last being capable of being removed by filtration.

(2) When fresh ox serum is passed through a Berkefeld filter and successive portions of the filtrate are examined, it is found that certain of the early fractions may contain antibody, but, owing to the absence of complement, are not lytic alone. Such filtrates are capable of sensitising guinea-pig red cells on contact at 37° C.—less effectively at room-temperature or in the ice-chest—so that the latter when washed and resuspended in salt solution undergo lysis on the addition of guinea-pig complement. Such filtrates lose their affinity for the red cells on standing for some hours at room-temperature or on heating at 53° C. for thirty minutes, although they retain to a great extent their antibody content. But the recent, unheated filtrate does not lose its sensitising power as the result of treatment with a complement absorbing agent, such as a suspension of staphylococci. The addition of fresh guinea-pig serum in sublytic doses to heated ox serum does not enhance the sensitising power of the latter. Thus there is no evidence that the combination of antibody with the red corpuscles (sensitisation) is assisted by a complement-like body. No evidence has been obtained in favour of the view of Ehrlich and Sachs that the phenomena in question imply or are due to a direct union of complement with antibody.

(3) As regards the relationship of "conglutination" to hæmolysis, it has been found that while certain portions of filtrate contain antibody and also cause agglutination, the portion of filtrate preceding these possesses sensitising power, but lacks agglutinating action either alone or in the presence of suitable complement, such as that of the pig. Hence conglutinin is not essential for the lysis of guinea-pig corpuscles by complement and the natural antibody contained in ox serum. The experiments show that the affinity of this antibody for guinea-pig corpuscles is interfered with by other constituents of the serum. When the latter are altered or removed by filtration the antibody is then capable of sensitising the red cells.

(4) Conglutination appears to depend on some distinctive property of ox serum, acting together with antibody and complement, as Bordet and his co-workers believe. Thus portions of filtrate of fresh ox serum, which by themselves are not lytic, but which cause agglutination, lose the latter action after treatment with a suspension of staphylococci, or after heating for thirty minutes at 45° to 48° C. The action is restored by adding minute amounts of fresh pig serum.

(5) Although the lytic antibody of filtrates is fairly stable, agglutinating properties rapidly deteriorate on standing at room-temperature or on ice. Neither this deterioration nor that of the sensitising property is to be ascribed to the slight changes in the hydrogen-ion concentration of the serum due to contact with the filter.

REFERENCES.

1. BORDET AND GAY "On the Relations of Sensitizers to Alexin"; see Bordet's *Studies in Immunity* (trans. F. P. Gay), New York, 1909, p. 363.
2. BORDET AND STRENG . . . "The Phenomena of Adsorption and the Conglutinin of Bovine Serum"; see Bordet's *Studies in Immunity* (trans. F. P. Gay), New York, 1909, p. 440.
3. BROWNING, C. H. . . . "Agglutination u. Komplementschwund," *Wien. klin. Wchnschr.*, 1906, Jahrg. xix. S. 441.
4. BROWNING, C. H., AND MACKIE, T. J. "Über d. Beziehungen d. Komplementwirkung d. frischen Serums bei d. Aktivierung d. Immunkörper und d. Kobragiftes," *Biochem. Ztschr.*, Berlin, 1912, Bd. xliii. S. 229.
5. EHRLICH AND MORGENROTH "Studies on Hæmolysins, Fourth communication"; see Ehrlich's *Collected Studies on Immunity* (trans. C. Bolduan), New York, 1910, p. 56.
6. EHRLICH AND MORGENROTH "Studies on Hæmolysins, Sixth communication"; see Ehrlich's *Collected Studies on Immunity*, New York, 1910, p. 88.
7. EHRLICH AND SACHS. . . "Concerning the Mechanism of the Action of Amboceptors," (2); see Ehrlich's *Collected Studies on Immunity*, New York, 1910, p. 217.

8. KLOMER, J. A. "The Nature of Thermolabile Hemolysins,"
Journ. of Immunology, Baltimore, 1919, vol.
iv. p. 403.
9. KOSAKAI "The Effect of Osmic Acid and other Agents
on the Affinity of Receptors for Immune
Bodies and on their Antigenic Properties,"
Journ. Path. and Bacteriol., Cambridge,
1920, vol. xxiii. p. 425.
10. LESCHLY, W. "Versuche über Konglutination," *Ztschr. f.*
Immunitätsforsch, Jena, 1916 (Orig.), Bd.
xxv. S. 219.
11. MUIR, R., AND BROWNING, "On Chemical Combination and Toxic Action
C. H. as Exemplified in Hæmolytic Sera," *Proc.*
Roy. Soc., London, 1905, B, vol. lxxiv. p. 298.
12. MUIR, R., AND BROWNING, "On the Action of Complement as Agglutinin,"
C. H. *Journ. Hyg.*, Cambridge, 1906, vol. vi. p. 20.
13. MUIR, R., AND BROWNING, "On the Filtration of Serum Complement,"
C. H. *Journ. Path. and Bacteriol.*, Cambridge, 1909,
vol. xiii. p. 232.
14. NEUFELD, L. "Ueber d. Einfluss d. Toxizität d. Komplements
und d. Hämagglutination auf d. Ablauf d.
Hämolyse," *Ztschr. f. Immunitätsforsch.*,
Jena, 1918 (Orig.), Bd. xxvii. S. 373.
15. SACHS AND BAUER "The Joint Action of Several Amboceptors
in Hæmolysis and their Relation to the
Complements" ; see Ehrlich's *Collected*
Studies on Immunity (trans. C. Bolduan),
New York, 1910, p. 616.
16. SCHMIDT, H. "Studies on the Berkefeld-filtration of Comple-
ment," *Journ. Hyg.*, Cambridge, 1914, vol.
xiv. p. 437.