THE THERMOLABILITY OF COMPLEMENT, IN RELATION TO THE HYDROGEN ION CONCENTRATION.

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That property of fresh blood serum by which it is able to take part in, and complete various immunological reactions is known as complement. The thermolability of this function is well known. In respect to the separated fractions, that contained in the euglobulin fraction of the serum and known as mid-piece, and that contained in the pseudoglobulin and albumin fraction and referred to as end-piece, Ferrata[1] found only the end-piece thermolabile. Subsequent observers have uniformly found both fractions thermolabile. A number of investigators[2, 3, 4] have noted that the mid-piece is more resistant when heated in whole serum than when isolated. Leschly[5] found moreover that the mid-piece, apparently inactivated by brief heating to 56°C. was able to complete hemolysis when added separately to the sensitized cells, the addition of end-piece being made after an interval, so that under these conditions there is not a permanent destruction, but the development of the modification described by Brand.[6]

With regard to the effect of the chemical reaction it has been known since the observations of Ehrlich and Morgenroth[7] and Ehrlich and Sachs[8] that the addition of considerable amounts of acid or alkali

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[1] Ferrata, A., *Berl. Klin. Woch.*, 1907, xliv, 366.

[2] Freidmann, U., *Z. Hyg. und Infektionskrankh.*, 1910, lxvii, 279.

[3] Marks, H. K., *Z. Immunitätsforsch. Orig.*, 1911, xi, 18.

[4] Mutermilch *Compt. Rend Soc. de Biol.*, 1911, lxx, 577.

[5] Leschly, W., *Z. Immunitätsforsch. Orig.*, 1916, xxv, 44.

[6] Brand, O., *Berl. Klin. Woch.*, 1907, xliv, 1075.

[7] Ehrlich, R. and Morgenroth, J., *Berl. Klin. Woch.*, 1899, xxxvi, 481.

[8] Ehrlich, R., and Sachs, H., *Berl. Klin. Woch.*, 1902, xxxix, 297.

permanently inactivate complement. The destructive or inhibitory effect of slight degrees of acidity or alkalinity has been noted by a number of later workers. Liefmann and Cohn,[9] and Guggenheimer[10] found that the isolated mid-piece is more affected by the chemical reaction than is the end-piece; both fractions are more affected by acid than by alkali. Michaelis and Skwirsky[11] and Leschly[5] have worked with definite H ion concentrations, but their results do not relate to the present problem. Brooks[12] has recently defined the limits of pH value of the acidity beyond which complement is permanently inactivated.

The salt concentration of the solution has been found to affect complement in a way which appears to be quite different from that in which the two fractions of complement are separated by dilution with distilled water and dialysis, as in the original method of Ferrata.[1] Sachs and Teruuchi[13] found that when fresh serum is diluted 1 to 10 with distilled water a permanent inactivation occurs, and takes place more rapidly at 37°C. than at lower temperatures.

In the present work the degree of complementary activity retained by fresh guinea pig serum after being heated to various temperatures in solution in distilled water or in saline solution, at definite H ion concentrations, has been determined by a modification of the method of Brooks.[14] This consisted in measuring in a colorimeter to provide a standard for comparison the percentage of hemolysis given by the following amounts: 1, 0.8, 0.6, 0.4, 0.3, 0.2, 0.1, and 0.05 cc. of a dilution of each specimen of serum in saline solution. This dilution was first made 1 to 20, and then slightly increased to correspond exactly to the final dilution of the samples which were heated under varying conditions of temperature and reaction. The degrees of hemolysis thus observed were plotted as ordinates against the amounts of complement dilution as abscissæ. From the curve so obtained the relative efficiency or activity of each

[9] Leifmann H., and Cohn, M., *Z. Immunitätsforsch. Orig.*, 1910, vi, 562.
[10] Guggenheimer, H., *Z. Immunitätsforsch. Orig.*, 1911, xi, 393.
[11] Michaelis, L. and Skwirsky, P., *Z. Immunitätsforsch. Orig.*, 1910, vi, 357, 629
[12] Brooks S. C. ,*J. Gen. Physiol.*, 1919–20, 185.
[13] Sachs, H., and Teruuchi, Y., *Berl. Klin. Woch.*, 1907 xliv, 467.
[14] Brooks, S. C., *J Med. Res.*, 1919–20, xli, 399.

heated sample can be obtained by reading the abscissa at which the percentage of hemolysis given by 1 cc. of the diluted and heated specimen intersects the base-line curve. The various corrections used by Brooks were not made. By the use of this principle as Brooks has shown, one compares amounts which produce like results, and can thereby obtain the actual ratio between the unheated control and the heated sample with respect to the complementary function under consideration. For each complete experiment there was used one stock emulsion of 5 per cent sheep cells sensitized with two units of sensitizer. The total volume in each tube of the titration and of the test was 5 cc. before the addition of acid or alkali.

The H ion concentration was determined by diluting three 2.5 cc. portions of each specimen of serum to 5 cc. with distilled water or saline, according to the medium chosen, adding methyl red and bromthymol blue separately to two of the tubes and determining by comparison with a standard series the H ion concentration reached after the addition of successive drops of N/40 HCl in equal number to each tube. The third tube served as a color screen after each addition of acid.[15] For each specimen of the isolated fractions of complement a similar titration was carried out, using for the end-piece N/40 and for the mid-piece N/160 HCl and NaOH.

The partition of the complement into the fractions was carried out by bringing the fresh serum diluted 1 to 20 with cold distilled water to a pH between 6.2 and 6.4, using for this purpose the necessary amount of HCl as calculated from the titration described above. This narrow range of reaction is that which was found optimal for the precipitation of the euglobulin under these conditions. A titration was found necessary for each specimen of serum because of differences in the amount of acid required to bring the pH to the desired reaction, and the blood of a single animal for the same reason was used for each experiment. The different methods which have been used for the partition of complement obviously depend upon the insolubility of the euglobulin within a narrow range of reaction

[15] Electrometric control of the colorimetric determinations indicate that the limit of error in the latter is pH 0.2. Between pH 5.8 and 6.5 the error is consistent in sign and lies between +0.15 and +0.10, the electrometric pH is more acid. No corrections have been made on the curves given here.

in a salt-poor medium. After bringing the serum dilution to the desired reaction it was left standing 1 hour on ice and the flocculent precipitate brought down by centifugation. The supernatant fluid was decanted, the inner walls of the tube wiped dry with a clean cloth and the sediment representing the mid-piece used at once or after a single washing with distilled water of pH 6.0. The supernatant fluid from the first sedimentation was used as the end-piece dilution. The final preparation of mid-piece was made by carefully emulsifying the sediment in distilled water, or by subsequently bringing it into solution by the addition of NaOH to pH 7.4.

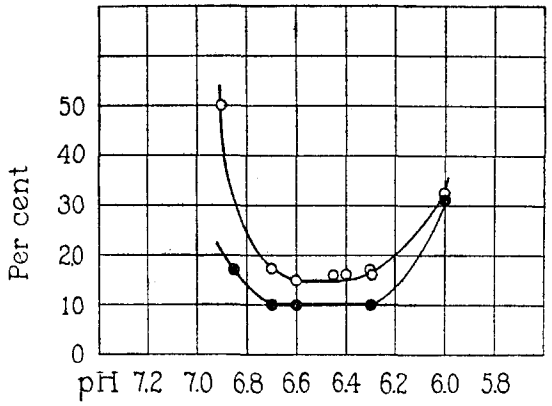

FIG. 1. The complementary activity of the supernatant fluid alone, after precipitation of the euglobulin at different reactions. One experiment is represented by the lower curve and two by the upper curve.

Many workers have had difficulty in separating completely the fractions of complement. The extent to which the completeness of separation depends upon the hydrogen ion concentration at which the separation takes place, is shown by the curve, Fig. 1. This records the complementary activity of the supernatant fluid alone separated at different reactions from the euglobulin sediment.

After adding the amounts of acid or alkali necessary to produce the desired reaction, to a series of tubes containing the different complement dilutions, the tubes were heated in all cases for 10 minutes in a water bath, then cooled by placing in ice water. The fluids in the tubes were all brought to the same reaction (pH 7.3 in the case of whole

serum, pH 6.3 in the case of the fractions) the volumes equalized, and where distilled water had been used as the diluent, isotonicity was restored by the addition of one-tenth volume of 8.5 per cent NaCl solution. The original dilution was 1 to 20 in all cases, and the fractions were used in the same corresponding dilution. Between manipulations the tubes were placed in ice water and protected from sunlight.

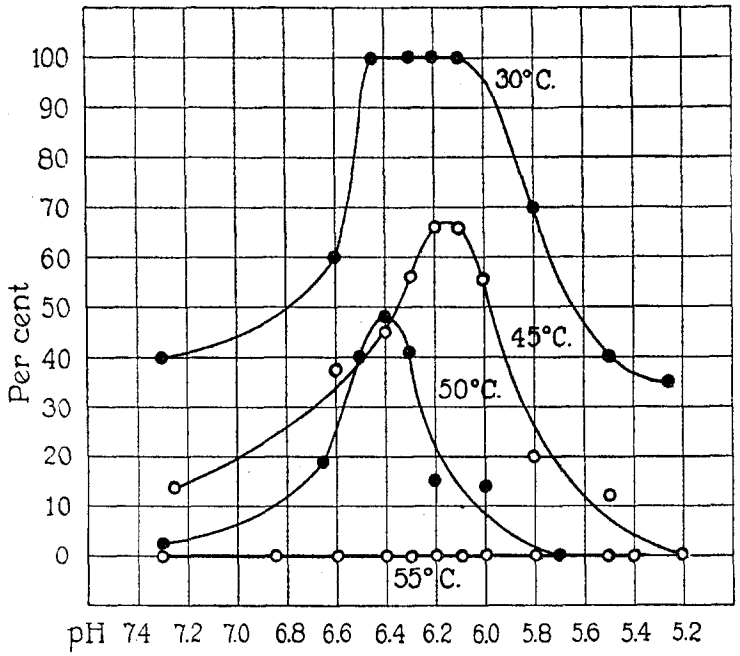

FIG. 2. The complementary activity retained by whole serum diluted in distilled water and heated at different temperatures, at varying reactions.

The complementary activity in percentages of that of the unheated control retained by whole serum diluted 1 to 20 in distilled water and heated at varying reactions at different temperatures, is shown in Fig. 2. The experiments recorded gave the highest values observed in many similar experiments. The value at any given pH relative to the unheated control varies with different specimens of serum, so that each curve is given to represent a single experiment. Although the amount of destruction increases with the temperature it is possible on account of this individual variation in thermolability,

which has been noted by all observers, to make accurate comparison only of the relative degrees of inactivation at the different reactions. In all experiments it was found that the destruction of complement is least at a point between pH 6.1 and 6.4. Within this narrow range from 20 per cent to 50 per cent of the original, activity may be retained when the complement is heated to 50°C. for 10 minutes. On either side of the optimal reaction the drop in activity is rapid. The curves run approximately parallel for all temperatures of heating. At 53°C. a very small degree of hemolytic activity is retained at the optimal reaction; when heated to 55°C. for 10 minutes all complementary power is lost, when referred to the small amount of sensitizer used here, at all reactions.

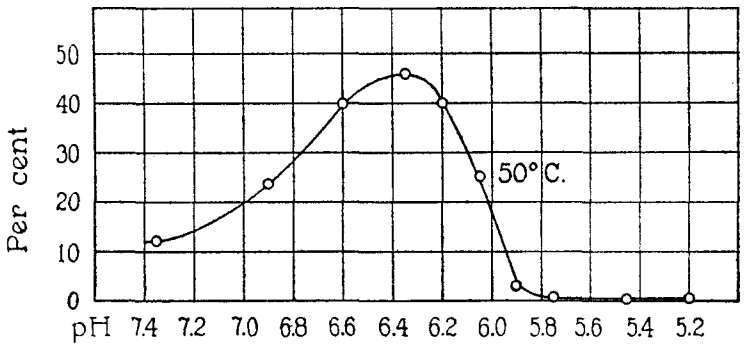

FIG. 3. The complementary activity of the euglobulin fraction in the presence of added end-piece, after heating whole serum to 50°C. in distilled water at the pH values given, and then separating the euglobulin.

If after heating the serum dilution the euglobulin is separated by bringing the reaction in all the tubes to pH 6.3 and removing the sediment by centrifugation, the complementary power of this euglobulin in the presence of the titer amount of end-piece follows the curve given in Fig. 3. It is evident then that the preservation of the complementary power at pH 6.1 to 6.4 depends upon preservation of the mid-piece function at these reactions.

A second maximum in the preservation of the complement was found in numerous experiments at pH 6.9, as shown in the lower curve in Fig. 4, from a typical experiment. The importance of this particular reaction was not appreciated in the early experiments

in which it was not included. It depends apparently upon the greater preservation of the end-piece function at this reaction. This will be referred to later. If the heated complement be added, without separation into its fractions, to sensitized cells together with unheated mid- and end-piece in separate series, the upper curves of Fig. 4 result. The two maxima observed at pH 6.9 and at pH 6.1 to 6.4 appear to be an expression of the quantitative relations between the two fractions, according to which the degree of hemolysis is in-

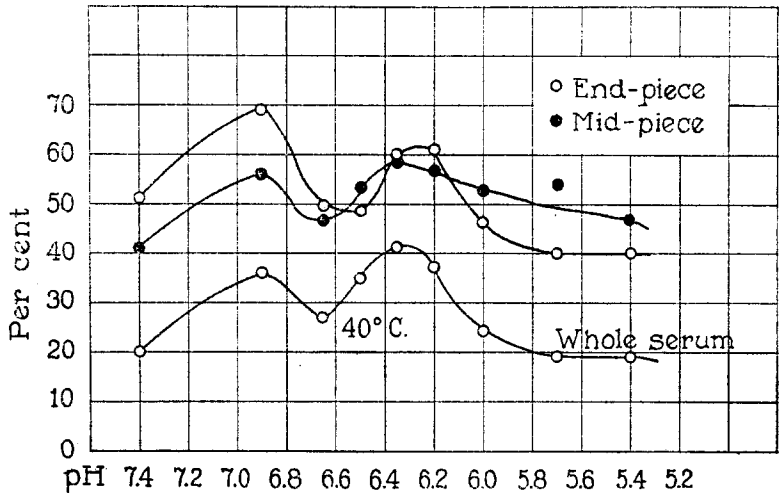

FIG. 4. The complementary activity of whole serum heated to 40°C. in distilled water dilution, by itself and in the presence of added mid- and end-piece. The curve marked end-piece is that given after the addition of the titer amount of mid-piece to each tube; that marked mid-piece is that given after the addition of the titer amount of end-piece to each tube.

creased if the amount of either mid- or end-piece is increased up to the titer value of each.[5]

The activity retained by the isolated end-piece fraction, freed from mid-piece by the precipitation of the euglobulin and heated in dilution in distilled water is shown in Fig. 5. In the experiments recorded here with the isolated fractions the original activity of the complement was completely restored on reuniting the unheated fractions, and the curves that show the effect of heating are essentially the same whether the activity is estimated from the base-line

curve given by the unsplit and unheated control or from that given by varying amounts of one fraction and constant amounts of the other fraction, in each tube of the control titration. It is evident that there is a broad optimal zone for the preservation of the end-piece function, with a reaction of about pH 6.9 as its central and highest point. At this reaction the end-piece is relatively thermostable as compared with whole complement.

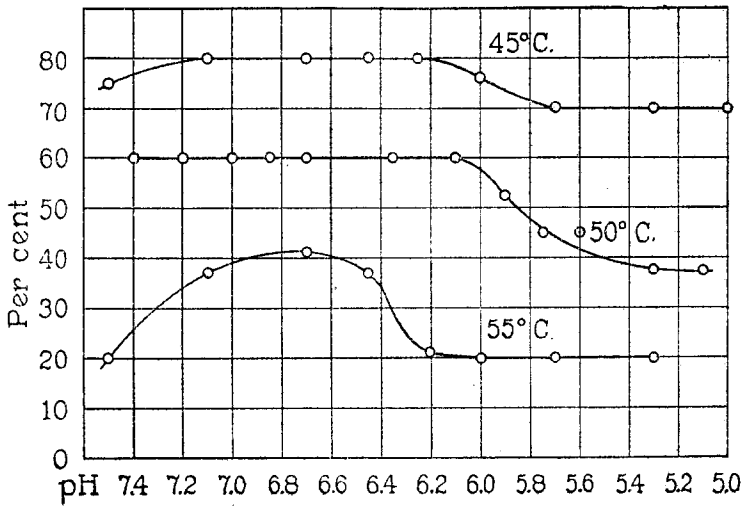

FIG. 5. The complementary activity in the presence of unheated mid-piece of the end-piece fraction heated separately in distilled water dilution at different reactions.

A similar relative independence of the H ion concentration as compared with whole complement is shown by the isolated mid-piece fraction, heated in distilled water. Two experiments at 50°C. are shown in Fig. 6. There is an optimal zone about pH 6.2 with a gradual fall on the alkaline side and a more rapid fall on the acid side of this point.

In the experiments with the isolated fractions the mid-piece whether heated or unheated was added first to the sensitized cells in order to avoid the peculiar modification described by Brand.[6] The curves represent therefore the degree of complete or irreversible inactivation of the fractions.

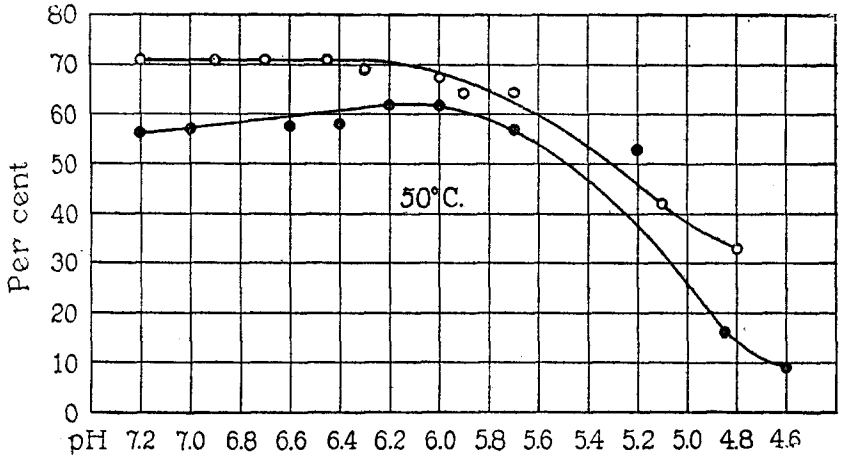

FIG. 6. The complementary activity in the presence of unheated end-piece of the euglobulin (mid-piece) fraction heated separately in distilled water at different reactions.

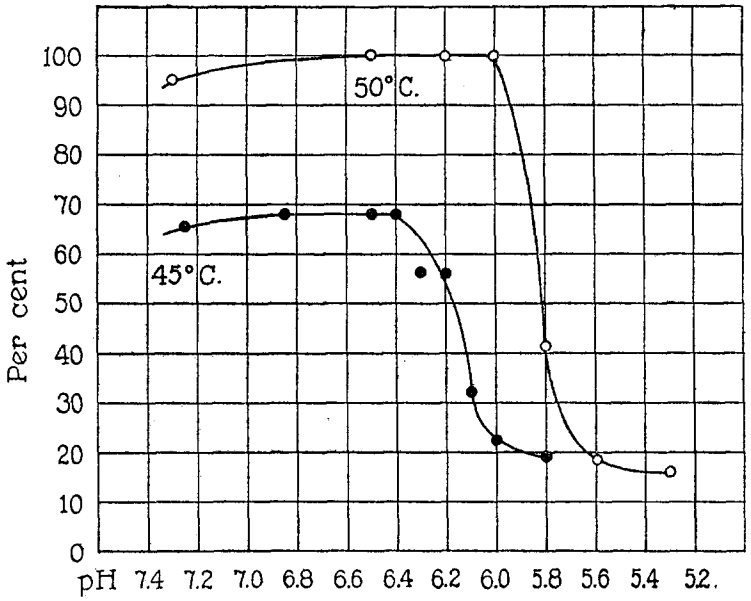

FIG. 7. The complementary activity of whole serum heated at different reactions in isotonic saline solution. The individual variation in thermolability is shown by the different maxima reached in the two experiments recorded here, the lower values being given in this case by the serum which was heated to the lower temperature.

The mid-piece function is thus better preserved when isolated than when heated in the presence of the other constituents of serum at all the reactions except those in the narrow zone pH 6.1 to 6.4, within which it is almost equally thermostable in both cases.

If fresh guinea pig serum is heated in dilution in saline solution instead of in distilled water it is evident from Fig. 7 that the complementary function is better preserved and is less affected by change in reaction than when heated in distilled water, at reactions on the alkaline side of pH 6.1 to 6.4. On the acid side of this point the loss in activity with increasing acidity is as great in the presence as in the absence of salt. The determination of the H ion concentration is affected with a greater error in the dilution of the serum in saline than in distilled water, when colorimetric methods are used. Nevertheless the turning-point observed is essentially the same as in the experiments in distilled water dilution, and corresponds with the point on the curve given by Brooks[12] at which the destruction of complement begins in saline solution when the temperature is kept at 10°C.

DISCUSSION.

The inactivation which complement undergoes when heated in distilled water dilution is closely related to the properties of the euglobulin fraction since this destruction is least at the reaction at which the euglobulin is least soluble. The chemical reaction involved in thermolability in which the euglobulin is necessarily one of the reacting substances is therefore determined by the chemical state in which the euglobulin exists.

It has been found in the case of a number of proteins that solubility and other physical properties are at a minimum at the H ion concentration characteristic of the isoelectric point. At this point the ionization of the protein either as an acid or as a base is at a minimum. If the euglobulin sediment from serum is washed with a large volume of distilled water and brought into solution again by the addition of NaOH to pH 7.4 it becomes least soluble on the addition of HCl between pH 5.1 and 5.7 (depending probably on its purity) and is isoelectric about pH 5.0. The latter determination was made electrometrically; the value corresponds closely with that given by Rona

and Michaelis[16] for serum euglobulin (about pH 5.2). When examined in whole serum however the euglobulin precipitates best at pH 6.3 as noted above and shows no movement in the electric field between pH 6.2 and 6.4. Under these conditions it exists therefore probably not as pure euglobulin but as a compound with some other substance of the serum.

Different compounds of a protein may differ in the position of the isoelectric point. When the reaction of red blood cell suspensions in saccharose solution is adjusted with NaOH and HCl the isoelectric point as determined by cataphoresis and the optimum for agglutination lie near pH 4.7;[17] in the presence of sodium acetate and sodium phthalate this critical point is found near pH 3.8.[18] The compound with a weak acid thus differs from the compound with a strong acid, as pointed out by Professor J. L. R. Morgan;[19] it is possible then that the value pH 6.1 to 6.4 represents the critical point in the ionization of a compound of the euglobulin with a weak base, in which state it exists in the serum or when dissolved or suspended in distilled water without washing. We may conclude then that it is chiefly or entirely the ionized fraction of the euglobulin which takes part in the reaction involved in thermolability.

The existence of a point of inflection in the thermostability of the end-piece at pH 6.9 suggests a similar interpretation in the case of this fraction. No other data are available however by which to identify the substance or compound concerned.

The difference in behavior of the fractions of complement when heated separately and when heated together suggests that during the process of thermoinactivation the ions of the euglobulin compound combine or interact chemically with substances contained in the pseudoglobulin and albumin fraction.

The protection against destruction afforded by the presence of NaCl on the alkaline side of pH 6.1 to 6.4 may be explained by the depression in ionization of the Na euglobulin compound existing

[16] Rona, P., and Michaelis, L., *Biochem Z.*, 1910, xxviii, 193.

[17] Coulter, C. B., *J. Gen. Physiol.* 1920–21, iii, 309.

[18] Unpublished experiments.

[19] Personal suggestion.

at these reactions[20] which is caused by the high concentration of the common Na ion. On the acid side of pH 6.1 to 6.4 the protein exists in amounts increasing with the acidity in the cationic condition. We should expect a similar depression in the ionization of the protein in this form to be caused by the excess of the common Cl ion, but since at these reactions the destruction of complement is as great in the presence as in the absence of salt it is apparent that the behavior of the protein as cation is different from that as anion. This conclusion was suggested by Brooks[12] from a somewhat different evaluation of similar data.

CONCLUSIONS.

1. The destruction which complement undergoes on being heated in dilution in distilled water is least at a reaction between pH 6.1 and 6.4. This depends upon the relative preservation of the mid-piece function at this point. This reaction represents probably the isoelectric point of a compound of the euglobulin with some substance present also in serum.

2. During the process of thermoinactivation it is chiefly or entirely the ions of this euglobulin compound which react, and these combine or interact with substances contained in the pseudoglobulin and albumin fraction.

3. The behavior of the euglobulin is different in the anionic and in the cationic condition, since on the acid side of pH 6.1 to 6.4 the destruction by heat increases as rapidly with the acidity in the presence as in the absence of NaCl. On the alkaline side of this point the presence of NaCl protects complement from destruction because of the depression in the ionization of the euglobulin.

[20] Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 39, 237, 363, 483, 559.