CXXX. CALCIUM AND COMPLEMENT ACTION.

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LIEBERMANN [1921] has expressed the view that the active complement responsible for immune haemolysis is none other than the soaps normally occurring in the serum. He postulates that the general haemolytic properties of these compounds are suppressed by other constituents of the serum, proteins and calcium compounds, so that haemolysis results only in the presence of the sensitising substance. Thus this author claims to be able to prepare an artificial complement by adding to heat-inactivated rabbit serum a methyl alcoholic solution of calcium oleate, the mixture obtained being haemolytic towards sensitised cells but having no effect on unsensitised cells. Similar solutions have been prepared by Freund [1923], who used an aqueous solution of calcium oleate and the globulin fraction of bovine serum. Schmidt [1924], however, has been unable to repeat these results and states that the solutions obtained have no specific action on sensitised cells. Liebermann himself appears to have experienced difficulty in this respect and attributes it partly to the impossibility of obtaining suitable methyl alcohol. Although he maintains that his artificial complement will give satisfactory results when used instead of natural complement in the Wassermann reaction, he admits that some improvement in the technique of preparation is necessary before it can be used extensively in practice.

With the exception of this work on the preparation of artificial complement, there is little evidence available of the possible rôle played by calcium compounds in complement action. Various authors (Wright and MacCallum [1922] and Purdy and Walbum [1922]) have shown that calcium salts exert an anticomplementary action, even small amounts inhibiting haemolysis, and it is possible that the occasional anticomplementary action of certain fractions of complement may be due in part to the small amount of calcium salts present. The amount of calcium normally present in serum is not sufficient to exert any powerful anticomplementary action in the whole serum, but the amount may be sufficient for the calcium to have some active part in the complement reaction.

The inability of other workers (including the present authors) to prepare artificial complement by Liebermann's method may be due to some slight

differences in technique and the question of the validity of Liebermann's views appears to warrant investigation along other lines. If this view of the nature of complement is correct, then the artificial complement, besides having a specific haemolytic action on sensitised red cells, should be heat-labile, and CO_2 and dilute acids should separate it into two inactive fractions which together have full complement activity. The splitting of complement by CO_2 has not yet been explained on any other grounds than the precipitation of the globulin fraction and it is of interest, therefore, to consider what would happen if complement consisted principally of calcium salts of fatty acids associated with the serum colloids. The calcium in serum is only partially dialysable, and the non-dialysable calcium is presumably in combination with proteins [Marrack and Thacker, 1926], fatty acids and perhaps other compounds. By passing in an excess of CO_2 , we should expect that the calcium would be split off from combination with these compounds and that it would appear as calcium bicarbonate in the solution. In the splitting of complement by this method, therefore, the globulin fraction should contain little or none of the calcium present in the serum. This we have found to be the case, for all the calcium invariably passes into the albumin fraction. Furthermore, if calcium oleate is added to heated rabbit serum as in Liebermann's preparation, and then subjected to CO_2 treatment all the added calcium is found in the albumin fraction. According to this view of the nature of complement, therefore, the splitting of complement by dilute acids can be explained on purely chemical lines, but in our opinion the problem is not quite so simple although this may be part of the true explanation.

If the active principle of complement is a calcium soap and if this substance or closely related compounds are solely responsible for the specific haemolytic properties, the protein and other serum colloids being concerned only indirectly, true splitting of complement should only be possible with acids which, like CO_2 , give a soluble calcium salt. Thus if CO_2 separates the complement into two fractions, a globulin precipitate containing the insoluble higher fatty acids, and a "soluble" albumin fraction containing calcium bicarbonate, all acids which give soluble calcium salts should have a similar effect, whereas acids such as sulphuric and oxalic would precipitate the calcium in the wrong fraction. Only those acids giving soluble calcium salts should therefore give complete separation of complement into two separately inactive fractions identical with those obtained by CO_2 treatment. A large number of experiments have been carried out with various acids and the fractions obtained compared with the normal mid-piece and end-piece of the CO_2 separation. The chemical separation obtained agrees in every respect with the theory, for all or practically all the calcium was found in the albumin fraction when CO_2 , hydrochloric or acetic acids were used and it was mainly in the globulin fraction in the case of oxalic acid. When sulphuric acid was used calcium was present in both fractions, mainly in the albumin fraction, a result which was expected since calcium sulphate is somewhat soluble. With

oxalic and sulphuric acids it was found that the best results were obtained when the acids were allowed to act for some considerable time (2 hours) and this was attributed to the formation of insoluble calcium salts on the non-ionised complex calcium compounds and the consequent time required for the removal of calcium from these compounds. The results with reference to complement activity showed a general support of the calcium soap view but suggested that, while this theory is partially correct, there are other factors to consider. The fractions obtained with acids giving soluble calcium salts appeared identical in all respects with those obtained with CO_2 , but in the case of sulphuric and oxalic acids, the albumin fractions, which contained less calcium, and that probably in a different form, had little or no completing action for fresh CO_2 -globulin fraction. In all cases the globulin fractions were able to activate the CO_2 -albumin fraction. The only differences appear therefore in the albumin fractions, those from acids giving soluble calcium salts having the power to complete CO_2 mid-piece, whilst those from acids giving insoluble calcium salts have no such power. The re-formation of the complement by addition of each end-piece to its respective mid-piece fraction also gave some support to this theory, for full complement power was regenerated in the case of hydrochloric, acetic and similar acids, but not in the case of sulphuric or oxalic acids. The results were not as definite, however, as would be the case if this calcium soap view covered the whole explanation and there are probably other factors concerned. We suggest, therefore, very tentatively, that if calcium or some other salt of a fatty acid is the active haemolysin, the whole of this substance may not be present as such in the original serum at the beginning of the process of haemolysis. If this is so, separation of the calcium soap present will take place in the manner indicated according to the nature of the acid used, but this does not take into consideration the presence of other substances which may be concerned and which may be responsible for the renewal of the supply of the active agent.

Liebermann's views on the nature of complement have also been investigated in the light of the facts known about the non-dialysable calcium in serum. According to the general view, this non-diffusible calcium is chiefly in combination with protein and the higher fatty acids, and there is probably some state of equilibrium between the diffusible and the non-diffusible calcium. Loeb and Steinberger [1924], for example, found that only part of the calcium would diffuse out into water, the amount being sometimes as low as 55 % of the total calcium, but when dialysed against 0.8 % NaCl at p_H 7.4 or HCl at p_H 2.5, all the calcium was diffusible. This complete removal of calcium by dialysis against physiological saline is not supported by other results and our own experiments do not agree with this view. As a result of dialysis experiments of varied nature we have found that the removal of all the dialysable calcium does not inactivate complement. The diffusible calcium does not function therefore in the complement reaction except perhaps in the maintenance of a certain amount of calcium in combination with

proteins and fatty acids. If the calcium in serum is necessary for immune haemolysis it is only that which is present in the non-dialysable form, and according to the calcium oleate theory, any mechanism which tended to decrease the amount of non-dialysable calcium would decrease the haemolytic power. Ultra-violet light has a destructive action on complement [Courmont, Nogier and Dufourt, 1913], and according to Clark [1923] ultra-violet radiations increase the proportion of dialysable calcium in serum, although a conflicting result has been obtained by Moritz [1925]. A possible explanation of the destruction of complement by ultra-violet light, however, is that the physical conditions of the protein components are altered, and if any decrease in non-diffusible calcium does take place it could be explained by the decreased ionisation of the proteins or decreased affinity for calcium ions.

Interesting results have been obtained by dialysing the separated mid-piece and end-piece fractions against water and 0.9 % NaCl. In order to have calcium in both fractions, the saline used in the experiments was made up from the commercial product which contained a known small amount of calcium salts. The separate mid-piece and end-piece fractions therefore both contained calcium; the mid-piece by virtue of that in the sodium chloride added, whilst the end-piece contained all the calcium originally present in the serum together with that in the saline. The calcium in the globulin or mid-piece fraction was practically non-dialysable, whilst that in the albumin fraction was partly dialysable. These fractions were also dialysed against the calcium-containing 0.9 % NaCl. The globulin fraction gained calcium in the process of dialysis, the concentration of calcium inside the sac being greater than that outside at the end of the process. The globulin fraction thus has the power of combining with inorganic calcium to form non-diffusible calcium compounds. The albumin fraction appears to have enough calcium present to supply the demands of the proteins, etc., and it loses diffusible calcium when dialysed against water or 0.9 % NaCl. In the "regeneration" of complement by the addition of the albumin fraction to the globulin fraction, the latter, which contains no calcium, will take it up from the former and will regenerate non-diffusible calcium compounds. Thus these facts are in no way antagonistic to Liebermann's view.

Several considerations have led us to investigate the possibility of calcium being the fourth component or relatively heat-stable factor inactivated by ammonia (see preceding paper), the main factor being that the splitting of complement by CO_2 results in the separation of all the calcium in the albumin fraction in which the fourth component is found. In addition, if Liebermann's view is correct, the calcium from the active calcium oleate would be in that fraction. The mechanism of the process of inactivation by ammonia could possibly be explained along these lines by the formation of a non-ionised double salt of calcium and ammonium, and possibly phosphate, which would disturb the equilibrium between the dialysable and non-dialysable calcium and lead to a diminution of the amount of calcium combined with protein and fatty acids.

Repeated experiments have shown, however, that no increase in dialysable calcium occurs during the process of inactivation by ammonia and this view does not appear to be the true explanation of the inactivation process. On the other hand, there is a fair amount of evidence to suggest that calcium does function actively in complement action and is connected with the fourth component. Over an extended period calcium estimations have been carried out with the fractions of complement, and these have been compared with the power of such fractions to reactivate ammonia-inactivated serum. A general parallelism has been found, and in practically every case there is correlation between calcium content and the amount of the fourth component present. Thus calcium-free mid-piece fractions do not contain this component, whilst the end-piece fraction, which contains practically all the calcium originally present in the serum, is always rich in this factor. In addition the albumin fractions obtained when sulphuric and oxalic acids are used to split the complement contain little or none of this component and these fractions contain less calcium than the CO₂-albumin fraction and that present probably in an altered condition. Attempts have been made to reactivate ammonia-treated serum by the addition of small amounts of calcium salts or by the use of saline containing small amounts of calcium, but these have been unsuccessful, as have all our attempts to replace the fourth component by calcium. More evidence on the exact nature of the calcium compounds present in serum may be required before we can reproduce the same conditions artificially once the equilibrium has been disturbed.

EXPERIMENTAL.

The details for the separation of complement by CO₂ and for the inactivation by ammonia are described in the previous papers. The calcium estimations were carried out by Tisdall's modification [1923] of the method of Kramer and Tisdall [1921], and the amount of non-dialysable calcium was estimated after dialysis through collodion sacs [Moritz, 1925] or through parchment thimbles. Physiological saline made from pure NaCl (A.R.) was used for all purposes unless otherwise stated.

Dialysable calcium and complement action.

Guinea-pig serum was dialysed against water in collodion sacs under a negative pressure and against water and 0.9 % NaCl in parchment thimbles. The sera free from all dialysable calcium were still haemolytic towards sensitised cells and all contained calcium.

Similarly end-piece fraction dialysed alone against water or 0.9 % NaCl retained part of its calcium and also its full power to activate fresh mid-piece. This dialysed end-piece, even when heated at 56°, still contains the fourth component and will reactivate ammonia-treated serum.

Exp. Neutralised end-piece (CO_2) was dialysed in parchment thimbles for 24 hours against water and then made isotonic with NaCl. 60 % of the calcium dialysed out (average of several determinations) but the solution still retained the power to activate fresh mid-piece and also ammonia-inactivated serum.

1.0 cc. NH_3 -inactivated serum	...	...	...	...	—
0.5 cc. „	+ 0.5 cc. 0.9 % NaCl	...	...	...	—
0.5 cc. „	+ 0.5 cc. dialysed end-piece	...	...	...	+ + +
0.5 cc. „	+ 0.5 cc. heated dialysed end-piece	...	...	...	+ +
0.5 cc. fresh mid-piece	+ 0.5 cc. dialysed end-piece	...	...	...	+ + +

The separation of complement by CO_2 and the distribution of calcium.

Calcium estimations with the separate mid-piece and end-piece fractions (CO_2) showed that practically all the calcium in the serum passes into the albumin fraction. The same result was obtained with rabbit serum and if calcium oleate had been added previously, as in the preparation of Liebermann's artificial complement, this added calcium was always found in the albumin fraction.

Calcium estimations (mg. per 100 cc. of serum or equivalent amount of the fractions).

Table I. (*Guinea-pig serum.*)

In guinea-pig serum	10.5	8.5	7.8	—	—	8.0	11.0	8.0	—
In mid-piece fraction (CO_2)	0	0	0.4	0	0	0	0.3	0	0
In end-piece fraction (CO_2)	10.0	8.5	7.6	9.2	6.0	8.5	10.5	8.5	12.0

Table II. (*Rabbit serum.*)

Rabbit serum	...	...	...	...	12.8	6.0	12.0
Mid-piece (CO_2)	...	...	...	...	0	0	0
End-piece (CO_2)	...	...	...	...	13.2	5.8	11.8
Rabbit serum + calcium oleate (serum X)	...	...	...	...	20.0	12.8	16.8
Mid-piece of serum X	...	...	...	...	0	0	0
End-piece of serum X	...	...	...	...	20.4	12.3	17.0

Separation of complement by various acids, the calcium distribution and the fourth factor content.

Different acids were added to serum to give the same resulting p_{H} as that produced by passing CO_2 through for 15 minutes as in the ordinary method of separation. The sera were diluted to 1 in 10 with distilled water, allowed to stand for 1–2 hours, and centrifuged. The fractions were adjusted to p_{H} 7.5 and made isotonic with serum by means of pure NaCl. The results varied but in general the separation with acetic acid, phosphoric acid and HCl ran parallel with that by CO_2 , while oxalic, sulphuric and, to a lesser extent, citric acids did not give the same results. Thus the separated fractions obtained with the latter series of acids were not active when added together and this we attributed to the differences in the distribution of the calcium. The following is an average result, the separate mid-piece and end-piece fractions being inactive in all cases even when used in double quantity.

Table III.

	CO ₂	Acetic acid	HCl	H ₂ SO ₄	Oxalic acid
0.5 cc. mid-piece + 0.5 cc. end-piece	++++	++++	++++	+	-
0.5 cc. CO ₂ mid-piece + 0.5 cc. different end-pieces	++++	++++	++++	++	-
0.5 cc. CO ₂ end-piece + 0.5 cc. different mid-pieces	++++	++++	++++	+++	+++

In nearly every experiment the mid-piece fractions from the oxalic acid and sulphuric acid separations had a slight anticomplementary action when tested with small amounts of guinea-pig serum and this is presumably due to the insoluble calcium salt precipitated on the globulin. The anticomplementary power, however, is not responsible for the inactivity of the "re-formed" complement in these cases, for these mid-piece fractions can fully activate fresh CO₂ end-piece.

The separated fractions were then tested with ammonia-inactivated serum (see previous paper) and the calcium content of each fraction was also determined. In general the power to reactivate the ammonia-treated serum was only evident in solutions containing calcium. The converse was not always true but the inability of the globulin fractions with H₂SO₄ and oxalic acid to function in this way can be attributed to the fact that the calcium is present as an insoluble salt. The occasional slight power of the oxalic and sulphuric acid end-pieces to reactivate this inactive serum may be due to the calcium still left in the solution or to calcium from other sources, and in this connection it must be remembered that calcium is always present in the red cell [Parnas and Jasinski, 1922] and will be liberated as soon as any haemolysis occurs.

To obtain the best distribution of the calcium with the different acids it was found necessary to allow them to act upon the serum for 2 hours. The acids thus appear to split up the complex calcium compounds slowly, especially where the acid gives an insoluble calcium salt.

Table IV.

	CO ₂		HCl		Oxalic acid		H ₂ SO ₄	
	mid-piece	end-piece	mid-piece	end-piece	mid-piece	end-piece	mid-piece	end-piece
Calcium content	0	7.4	0	7.5	6.0	1.6	1.2	6.2
(mg. per 100 cc. of	0	8.4	—	—	6.2	2.4	2.4	6.0
the original serum)	0	7.6	—	—	6.6	0.6	0.4	7.6
	0	9.2	0	9.0	8.6	0.6	2.8	6.6
Fourth component	None	Present	None	Present	None	None or trace	None	Trace

The inactivation by ammonia and dialysable calcium.

Serum was inactivated by ammonia (see preceding paper) and the amount of dialysable calcium determined after dialysis in collodion sacs [Moritz, 1925] or in parchment thimbles for varying periods of time. Control experiments

in which serum was treated with one-quarter of its volume of 0.9 % NaCl and incubated for the same time showed that no increase in dialysable calcium had taken place during the inactivation process.

Table V. *Calcium estimations (mg. per 100 cc. of serum or equivalent amount of solutions).*

Guinea-pig serum	...	...	...	13.0	10.8	5.6	—	—
Non-dialysable calcium in NH_3 -inactivated serum				7.0	3.0	3.1	5.8	3.9
Non-dialysable calcium in control			...	7.5	3.0	3.2	5.0	4.1

SUMMARY.

(1) The relationship between the calcium in serum and complement activity has been investigated. The dialysable calcium is not essential for complement action but may be concerned indirectly on account of the equilibrium between dialysable and non-dialysable calcium. The non-diffusible calcium appears to be connected in some way with complement activity.

(2) The calcium content of various fractions of serum obtained by using CO_2 and other acids and the power to reactivate ammonia-inactivated serum show some correlation. Attempts to reactivate ammonia-treated serum by the addition of calcium in various forms were however unsuccessful.

(3) When complement is split by CO_2 all the calcium passes into the albumin fraction. If calcium oleate is added all the added calcium is found in the albumin fraction. The possibility of the chemical separation of complement by dilute acids, if complement consists of calcium soaps as in Liebermann's theory, is discussed.

(4) Acids which give insoluble calcium salts, such as oxalic and sulphuric, distribute the calcium differently, calcium being found in both fractions, in the case of oxalic acid mainly in the globulin fraction. The fractions obtained when these acids are used are inactive themselves and the combined fractions have little or no activity. Hydrochloric, acetic and phosphoric acids behave like CO_2 .

(5) No alteration in dialysable calcium occurs during the inactivation by ammonia.

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