CXXIX. THE ACTION OF AMMONIA ON COMPLEMENT. THE FOURTH COMPONENT.

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(*Received August 21st, 1926.*)

During a study of the action of pancreatic extracts on complement [Wormall, Whitehead and Gordon, 1925], we became interested in the possible action of lipase, thinking that it might throw some light on the part played by fats and lipins in complement action. The chief difficulty was to obtain lipase preparations free from proteoclastic enzymes, for trypsin preparations have a destructive action on both the albumin and globulin fractions of serum. Eventually we used the enzyme preparation of Willstätter and Waldschmidt-Leitz [1923] which is stated to contain little or no trypsin and to have a powerful lipolytic activity. The preparation consists of a suspension of purified lipase in an ammoniacal phosphate solution containing glycerol, and the results obtained by its action on complement indicated that the destruction of a hitherto unknown factor had occurred. A control experiment, however, showed that the boiled enzyme solution had the same function, and by a process of elimination it was found that the active agent was really the ammonia. In effect, the addition of ammonia (or ammonium salts and alkali) to complement and incubation at 37° for 1–2 hours, destroyed the power to haemolyse sensitised red blood cells. The activity of the serum could be restored by the addition of complement which had been heated to 56° and which was by itself inactive. The ammonia thus appeared to have destroyed some relatively heat-stable component of complement.

The components of complement which are known up to the present, and which can be separated from each other, are (1) the globulin component (mid-piece), (2) the albumin component (end-piece)—both heat-labile, (3) a relatively heat-stable third component. The third component is usually associated almost entirely with the globulin fraction, but on occasion small quantities are found in the albumin fraction. By the action of CO_2 and dilute acids on complement two fractions may be obtained: the globulin fraction containing a globulin component and most or all of the third component, and an albumin fraction (containing an albumin component). The two fractions are inactive separately but together form a system practically as active as the original serum. By the action of yeast or of zymin, a preparation which is

stable and which gives more satisfactory results than yeast [Whitehead, Gordon and Wormall, 1925], the third component can be removed and the serum becomes inactive because it contains the albumin and globulin components but no heat-stable third component. This inactive zymin-treated serum can be reactivated by the addition of guinea-pig serum heated to 56° for half an hour or by the globulin fraction heated in the same way, these heated solutions containing none of the heat-labile protein constituents. Thus it has been shown that at least three components are necessary for the complete complement action.

Brooks [1920] considers that the protein constituents of serum play little part in the process and that there is only one active substance produced from a precursor which may resemble lecithin, but he states that the activity of this lytic substance may be dependent on the state of the serum colloids. In our opinion this view does not emphasise sufficiently the rôle played by the proteins, which, even if not actively concerned, act as carriers of the active substances. Any destruction of the protein components causes loss in activity as shown by the action of trypsin [Michaelis and Skwirsky, 1910; Wormall, Whitehead and Gordon, 1925], or by the destructive action of acids whenever the p_H falls below that of the isoelectric point of the globulin [Brooks, 1920]. Therefore since mid-piece and end-piece both contain heat-labile constituents which appear to possess the general properties of proteins, it is necessary to include these protein constituents when considering the nature of complement. Until more definite evidence is forthcoming, it is sufficient for ordinary purposes to regard them as globulin and albumin.

As has been stated, guinea-pig serum can be inactivated by treatment with zymin or small amounts of ammonia or ammonium salts and both the inactive sera so produced can be reactivated by the addition of serum which has been heated to 56° and which is itself devoid of complement power. Further examination, however, shows that the inactive sera are not identical. Firstly, the heat-stable factor which is needed to reactivate zymin-treated serum has been shown to be associated mainly with the mid-piece of complement; the factor which will reactivate ammonia-treated serum is present mainly in the end-piece fraction. Secondly, ammonia- and zymin-treated sera will mutually reactivate one another, showing that each is deficient in some factor which the other can supply. It follows that two relatively heat-stable components of complement are involved and we must therefore postulate four components in the original complement; globulin and albumin (mid-piece and end-piece) heat-labile components; the third component removed or inactivated by zymin, and the fourth component destroyed or inactivated by ammonia treatment. It does not of course necessarily follow that the four substances are separate and distinct in the original serum, for the process of splitting complement by dilute acids may divide some components which were present originally in combination with each other. Recombination of these separated components would presumably take place when complement was reconstituted by the

addition of end-piece to mid-piece. The significance of this distinction is made evident in the following paper.

The CO_2 method of splitting complement does not always result in a clear-cut separation of the two heat-stable factors. It has already been shown that the third component is not always present in mid-piece only and similarly the fourth component, while present mainly in the end-piece, sometimes occurs in small quantities in the mid-piece. Consequently, while both mid- and end-pieces when fresh will often activate ammonia-treated serum, the amount of fourth component in mid-piece is so small that it is destroyed by heating for half an hour at 56°, so that of the heated fractions, only end-piece is effective.

After observing the main facts of inactivation by ammonia we were next concerned with attempts to elucidate the mechanism of the reaction. Other alkalis, such as caustic soda, were tried and it was found that their action was entirely different. Serum brought to the same p_H as the serum under treatment with ammonia, by addition of NaOH, and incubated in the same way, did not show a similar inactivation. In greater amounts or with a longer period of incubation there was a general destruction of complement which was not limited as with ammonia to inactivation of a heat-stable component. That is to say, an inactive serum produced by the action of NaOH could not be re-activated by the addition of any single component of fresh complement. The action of ammonia is therefore specific and not due simply to its alkalinity.

Ammonium salts were only effective when the p_H of the serum was 7 or over and the action was most pronounced at p_H 8–10. Any higher p_H (the reaction being adjusted with NaOH) resulted in a general destruction of the complement, identical with that observed when NaOH alone was used. Ammonia itself acted like other alkalis and destroyed the whole complement when added in amounts sufficient to raise the p_H beyond 10 or when the serum was incubated for long periods.

Other compounds containing NH_2 groups were then tried to see whether the action was due solely to that grouping. Amino-acids (glycine and alanine) and urea had no effect on complement in equivalent and in much larger amounts. Methylamine and ethylamine had the same effect as ammonia and it seems probable that the specific reaction is limited to ammonia and amines since the inactivation was not effected by neutral amino-compounds.

Time and temperature factors were then considered. By varying the conditions of temperature it was found that the time taken for inactivation was decreased with rise of temperature from 0° to 37°. At temperatures near freezing point the inactivation process was very slow and could be detected only after several hours. At room temperatures the velocity of the reaction was somewhat greater, but the best conditions were found to be the addition of 0·25 cc. of $N/6{\cdot}5$ NH_4OH to 1·0 cc. of guinea-pig serum and incubation for $1\frac{1}{2}$ hours at 37°. Under these conditions the action appeared to be specific towards the fourth component and remarkably regular, very rarely failing to give an inactive serum lacking only the fourth component.

Experimental.

The experiments were all carried out in duplicate at Leeds, where ox cells were used, and at Newcastle with sheep cells, and the results were identical at the two laboratories. The details of the haemolytic system were the same as previously used [Whitehead, Gordon and Wormall, 1925]. Guinea-pig serum was the source of complement and the results after incubation for 1 hour in an incubator or ½ hour in a water-bath are indicated as follows:

+ + + +	complete haemolysis	+ + +	intermediate degrees
+ +	half haemolysis	+	,,
−	no haemolysis		

Preparation of mid-piece and end-piece.

This was effected as described before [Whitehead, Gordon and Wormall, 1925] and in all experiments the excess of CO_2 was removed from the end-piece by evacuation and both fractions were adjusted to p_H 7·5.

The inactivation of complement by ammonia.

The inactivation described here was first obtained by using preparations of lipase prepared by Willstätter's method, but by elimination it was found that ammonia was the agent concerned and therefore an equivalent amount of ammonia was used. To 1 cc. of guinea-pig serum 0·25 cc. of $N/6{\cdot}5$ NH_4OH was added, the mixture shaken and incubated for 1½ hours at 37°. The serum was then adjusted, neutralised with $N/10$ HCl to p_H 7·5 and diluted to 10 cc. with physiological saline. This liquid (A) had no complete complement action itself but haemolysed sensitised cells if end-piece, end-piece heated at 56° for ½ hour, or heated guinea-pig serum was added.

Table I.

1·0 cc.	A	—	−
0·5 cc.	A	+0·5 cc. 0·9 % NaCl	−
0·5 cc.	A	+0·5 cc. mid-piece	− or +
0·5 cc.	A	+0·5 cc. heated mid-piece	−
0·5 cc.	A	+0·5 cc. end-piece	+ + + +
0·5 cc.	A	+0·5 cc. heated end-piece	+ + + +
0·5 cc.	A	+0·5 cc. heated complement	+ + + +

Solution A and all other solutions which had no complement action were tested for anticomplementary power. None of these solutions was anticomplementary and in addition, an amount of ammonium salts equivalent to or much greater than that present in solution A was likewise devoid of anticomplementary action. Thus the inactivity of A and similar solutions is due to the absence of some essential constituent or constituents.

Note. In the above and in all the experiments described in this paper the mid-piece and end-piece fractions were separately inactive but when mixed together they had a specific haemolytic action on sensitised cells almost as powerful as an equivalent amount of the original guinea-pig serum. The heated solutions were obtained by heating the fractions or the serum in a water-bath at 56° for 30 minutes.

Differences between sera inactivated by ammonia and by zymin (or yeast).

Guinea-pig serum was inactivated by ammonia as described above, and by zymin as described previously [Whitehead, Gordon and Wormall, 1925]. The neutralised solutions were both inactive but together were completely active. Each inactivated serum could be activated by the addition of heated serum, but, whereas the ammonia-treated serum was rendered active by the unheated or heated end-piece fraction, the zymin-treated serum was activated by the unheated or heated mid-piece fraction. Neither serum had any anti-complementary action when tested with varying amounts of fresh guinea-pig serum.

Table II.

A = Ammonia-inactivated serum. Z = Zymin-inactivated serum.

1·0 cc. A –	1·0 cc. Z –		A	Z
0·5 cc. inactivated serum (A or Z)		+ 0·5 cc. 0·9 % NaCl	–	–
,,	,,	+ 0·5 cc. mid-piece	–	+ + + +
,,	,,	+ 0·5 cc. heated mid-piece	–	+ + + +
,,	,,	+ 0·5 cc. end-piece	+ + + +	+
,,	,,	+ 0·5 cc. heated end-piece	+ + + +	–
,,	,,	+ 0·5 cc. heated guinea-pig serum	+ + + +	+ + + +

Strong confirmatory evidence that the two inactivated sera are deficient in different heat-stable factors has been obtained from experiments with heated guinea-pig serum and heated pig serum. Pig serum, which is extremely rich in the third component, contains much less of the fourth component, whilst guinea-pig serum is rich in both factors. Ammonia acting upon heated guinea-pig serum destroys the fourth component but not the third component, and the same amount of ammonia has no apparent action on the third component of heated pig serum.

2 cc. guinea-pig serum and 2 cc. pig serum heated at 56° for 30 minutes.

(*a*) 1 cc. of each serum was diluted to 10 cc. with 0·9 % NaCl.

(*b*) 1 cc. of each undiluted heated serum was treated with 0·25 cc. $N/6{\cdot}5$ NH_4OH, incubated at 37° for 2 hours, neutralised and diluted with 0·9 % NaCl to 10 cc.

Table III.

A and Z as in Table II.

			A	Z
0·5 cc. inactivated serum (A or Z)		+ 0·5 cc. heated guinea-pig serum (*a*)	+ + + +	+ + + +
,,	,,	+ 0·5 cc. NH_3-treated heated guinea-pig serum (*b*)	–	+ + + +
,,	,,	+ 0·5 cc. heated pig serum (*a*)	+	+ + + +
,,	,,	+ 0·5 cc. NH_3-treated heated pig serum (*b*)	–	+ + + +

Progress of the process of inactivation by ammonia and the influence of temperature.

Serum was treated with one-quarter of its volume of $N/6{\cdot}5$ NH_4OH and divided into three portions. One was kept in ice, one allowed to stand at room temperature, and the third incubated at 37°. Samples were withdrawn from each at the beginning of the experiment and at various intervals of time. The

samples were adjusted immediately to p_H 7·5 and diluted with saline so that they were equivalent to 1 : 10 complement. They were tested for action on sensitised cells with and without the addition of heated end-piece and heated serum. Heated or unheated mid-piece had no activating power, *i.e.* contained no fourth component.

Table IV.

Temp.		Time of incubation (hours) 0	½	1	2	3	4
37°	1·0 cc. NH_3-treated serum	++++	−	−	−	−	−
	0·5 cc. „ +0·5 cc. 0·9 % NaCl ...	++++	−	−	−	−	−
	0·5 cc. „ +0·5 cc. heated end-piece	++++	++++	++++	++	.+.	−
	0·5 cc. „ +0·5 cc. heated serum	++++	+++	+++	+	+	−
20°	1·0 cc. NH_3-treated serum	++++	++++	−	−	−	−
	0·5 cc. „ +0·5 cc. 0·9 % NaCl ...	++++	++	−	−	−	−
	0·5 cc. „ +0·5 cc. heated end-piece	++++	++++	++++	+++	+++	+++
	0·5 cc. „ +0·5 cc. heated serum ...	++++	++++	++++	+++	++	.++
0°	1·0 cc. NH_3-treated serum	++++	++++	++++	+	−	−
	0·5 cc. „ +0·5 cc. 0·9 % NaCl ...	++++	++++	+++	−	−	−
	0·5 cc. „ +0·5 cc. heated end-piece	++++	++++	++++	++++	++++	++++
	0·5 cc. „ +0·5 cc. heated serum...	++++	++++	++++	++++	++++	++++

Specificity of the action of ammonia. Comparison with the action of sodium hydroxide.

(*a*) Serum was adjusted by means of $N/10$ HCl or $N/10$ NaOH to approximate p_H values ranging from 7 to 11. An amount of NH_4Cl equivalent to the NH_4OH used in the previous experiments was added and the solutions incubated at 37° for 1½ hours. The solutions were then neutralised and tested with the various fractions of complement as before. Controls were carried out with serum adjusted to the same p_H values but without NH_4Cl. The inactivation of fourth component was noticeable only in alkaline solutions and was most pronounced at p_H 8·5–10·0. Controls at the same reactions, without NH_4Cl showed no destruction of complement power. Above p_H 10·0, the serum with or without NH_4Cl had lost all haemolytic action and could not be reactivated by any heated or unheated complement fraction. Thus above this p_H the action appeared to be a general destruction of the whole of the complement, due to the alkali present.

Experiment. 1 cc. guinea-pig serum + $N/10$ HCl or $N/10$ NaOH to required p_H + 0·25 cc. $N/6·5$ NH_4Cl.

Control. 1 cc. guinea-pig serum + the same amount of HCl or NaOH + 0·25 cc. normal saline.

All solutions incubated at 37° for 1½ hours, adjusted to p_H 7·5 and diluted with normal saline to 10 cc.

Table V.

	p_H 7·0	7·5	8·0	8·5	9·0	10	11
Serum with NH_4Cl	++++	++++	++++	−	−	−	−
1·0 cc. serum	++++	++++	++	−	−	−	−
0·5 cc. serum +0·5 cc. saline	++++	++++	++++	++++	++++	++	−
„ +0·5 cc. heated end-piece ...	++++	++++	++++	++++	++++	++	−
„ +0·5 cc. heated serum (1 in 10)	7·0	7·5	8·0	8·5	9·0	10	11
Serum without NH_4Cl	++++	++++	++++	++++	++++	−	−
1·0 cc. serum	++++	++++	++++	++++	++++	−	−
0·5 cc. serum +0·5 cc. saline	++++	++++	++++	++++	++++	−	−
„ +0·5 cc. heated end-piece ...	++++	++++	++++	++++	++++	−	−
„ +0·5 cc. heated serum ...							

Note. If the solutions with NH_4Cl at p_H 7·5 and 8·0 were left in the incubator for longer periods, destruction of the fourth factor took place, but this inactivation was very slow, especially at p_H 7·5.

(*b*) Serum was treated with varying amounts of NH_4OH and a similar series with equal amounts of NaOH. Incubation and other details were carried out as before. In no case did any specific inactivation of the fourth component occur with NaOH and where inactivation did occur it was due to general destruction of the whole of the complement. In relatively small amounts NH_4OH was quite effective in removing the fourth component whereas an equal amount of NaOH had no action.

Table VI. *Amounts of alkali to* 1 *cc. of serum.*

			0·25 cc. *N*/6·5 NH_4OH	0·25 cc. *N*/13 NH_4OH	0·25 cc. *N*/26 NH_4OH
1·0 cc. treated serum 1/10			−	−	+ + + +
0·5 cc.	,,	+0·5 cc. saline	−	−	+ +
0·5 cc.	,,	+0·5 cc. heated mid-piece	−	−	+ +
0·5 cc.	,,	+0·5 cc. heated end-piece	+ + + +	+ + + +	+ + + +
0·5 cc.	,,	+0·5 cc. heated serum	+ + +	+ + + +	+ + + +
			0·25 cc. *N*/6·5 NaOH	0·25 cc. *N*/13 NaOH	0·25 cc. *N*/26 NaOH
1·0 cc. treated serum 1/10			−	+ + + +	+ + + +
0·5 cc.	,,	+0·5 cc. saline	−	+ + + +	+ + + +
0·5 cc.	,,	+0·5 cc. heated mid-piece	−	+ + + +	+ + + +
0·5 cc.	,,	+0·5 cc. heated end-piece	−	+ + + +	+ + + +
0·5 cc.	,,	+0·5 cc. heated serum	−	+ + + +	+ + + +

General properties of the fourth component.

(*a*) This component is more stable at 56° than the two protein factors and in this respect resembles the third component. End-piece fraction if heated at 56° for ½ hour still contains the bulk of the fourth component although the albumin component has been destroyed. More prolonged heating slowly destroys the fourth component and after 1 hour at 56° there is little or none left in the solution. Like the third component, the fourth component is destroyed by heating for ½ hour at 66°.

(*b*) The fourth component is present mainly, if not entirely, in the end-piece and is therefore not precipitated by CO_2. It is non-dialysable since it is present in serum or end-piece which has been dialysed against water or saline in a parchment or collodion membrane for 24 hours. It is not removed from serum by treatment with zymin or yeast.

(*c*) It is destroyed when serum is made definitely acid or alkaline and incubated at 37° for 2 hours.

Summary.

(1) Small quantities of NH_3 have a destructive action on complement, removing a factor hitherto unrecorded. This component and the three other components previously recognised, must all be present for complete complement activity. Haemolysis of sensitised red cells only takes place therefore in the presence of two heat-labile protein components and two relatively heat-stable components.

(2) The fourth factor is relatively heat-stable and is associated with the albumin fraction. Ammonia-inactivated serum can thus be reactivated by the addition of heated serum or by heated or unheated albumin fraction.

(3) The fourth component is not identical with the third component which is removed by yeast or zymin, for the two components are associated with different fractions after the splitting of complement by dilute acids. Also, serum inactivated by zymin will reactivate ammonia-inactivated serum.

(4) The inactivation process is not immediate, but after a given time, depending on temperature and strength of ammonia, the fourth component is destroyed, leaving the other three components intact. At a later period the other components are destroyed owing to the alkalinity of the solution.

(5) Other alkalis have no similar specific action on the fourth component. Ammonium salts only act when the serum is previously adjusted to an alkaline p_H, the best reactions being above p_H 8·0 and below p_H 10·0. Thus free NH_3 is necessary for the inactivation process.

(6) The effect of temperature is very pronounced. The velocity at 37° is at least four times that at 0°.

(7) This new component is non-dialysable and not destroyed by heating at 56° for ½ hour, but it is readily destroyed at 66°. It is also destroyed by the action of dilute acids and alkalis at 37°.

We should like to express our thanks to Dr B. S. Platt and Mr Dawson for the solutions of lipase provided, and to the Medical Research Council for a grant in aid of two of the authors (J. G. and A. W.).

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