XCII. THE "THIRD COMPONENT" OR HEAT-STABLE FACTOR OF COMPLEMENT.

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THE separation of complement by various methods of protein precipitation into mid-piece and end-piece, or globulin and albumin fractions, has been well established.

Ferrata [1907] and Brand [1907] effected the separation by dialysis, Liefmann [1909] by saturation with CO_2 , and Sachs and Altmann [1909] by the use of dilute hydrochloric acid. Although the two fractions may be present as a single component in the original serum, the method of separation by dialysis appears to support the view that they are separate entities from the first; other evidence is in favour of some loose chemical or physical union between them. On the other hand, Browning and Mackie [1913] contend that the so-called mid-piece and end-piece fractions do not represent constant entities, basing this view on inability to get perfect separation on every occasion by the CO_2 method of Liefmann and on the results given by their ammonium sulphate separation. It seems, however, too exacting to expect the simple methods of protein separation to give results comparable with those of inorganic methods, although our experience of these methods, especially Liefmann's, has been rather more satisfactory. We find in almost every experiment that the separate fractions give practically no complement action whatever even when quantities corresponding to four times the minimum haemolytic dose (M.H.D.) are used, whereas, when combined, they show practically the same activity as the original complement.

Von Dungern [1900] was the first to describe a phenomenon whereby complement is inactivated by treatment with yeast cells. His observation was confirmed by Ehrlich and Sachs [1902], and Coca [1914] further showed that the inactivation was due to the removal of a relatively heat-stable component and not to the addition of an inhibiting factor. He found that yeast-treated complement could be reactivated by the addition of normal guinea-pig serum which had been inactivated by heating at 56° , or of either the mid-piece or end-piece. He also concluded that a similar inactivation of complement by means of cobra venom, observed by Braun [1911], Omorokow

[1911] and Ritz [1912], was due, not to absorption of the heat-stable component as in the case of yeast, but to inhibition of the function of this factor. He observed that cobra-venom-inactivated serum could at times supply the heat-stable factor to yeast-inactivated serum. Browning and Mackie [1914] working with cobra-venom-inactivated serum found that the heat-stable or third component was present in the mid-piece but not in the end-piece. Jonas [1913] working with pigs' serum found that this serum was particularly rich in third component, which was present in both mid-piece and end-piece. Moore [1919], Coca [1920] and Hyde [1923] reported the discovery of a race of guinea-pigs whose complement was deficient in third component. The serum from these guinea-pigs showed practically no complement activity, but could be activated by the addition of small amounts of heated serum in the same manner as yeast-treated serum.

Although the theories of complement action assuming the presence of mid-piece and end-piece have gained general acceptance, the presence of the third component in complement seems to be doubted by many observers. Thus references are made to the "so-called" third component and at other times the facts relating to this factor are disregarded. Browning and Mackie [1913] consider that it is doubtful whether the assumption of a third component can explain all the phenomena and conclude that complement power is due to the interaction of many factors. Brooks [1920], on the other hand, considers that only one haemolytic substance is concerned, which is constantly being formed from some precursor (probably a lecithin), but he admits that this lytic substance is dependent for its action upon the state of the serum proteins. The importance of the protein constituents in serum is emphasised by the experiments of Jacoby and Schütze [1910] on inactivation by shaking, and by those of Michaelis and Skwirsky [1910, 2] and the present authors [1925] on the destruction of part of complement by proteolytic enzymes. There seems, indeed, sufficient evidence to warrant the view that the serum proteins play a much greater part than is suggested by Brooks.

The objects of the present work were:

- (i) A fuller study of the action of yeast in removing third component.
- (ii) An attempt to determine the actual nature of the process and to obtain a more satisfactory and constant agent than yeast.
- (iii) To determine whether the third component is definitely associated, chemically or physically, with either of the two well-established protein fractions.

Dr R. R. Hyde of The Johns Hopkins University, Baltimore, kindly sent us some dried Fleischmann's yeast, which he recommended as an inactivating agent and this proved to be much more efficient than ordinary baker's yeast. Both yeasts, however, inactivated guinea-pig serum when used in excess, so that a paste was formed. The serum obtained by centrifuging the mixture after incubation for 2-3 hours was usually quite devoid of complement action and its activity could be restored by adding small amounts of guinea-pig

serum inactivated by heating at 56°. The method was, however, by no means certain, since much depended on the amount of yeast used and the time of incubation. The only way to obtain an inactive serum with certainty was to prepare a large quantity of yeast-serum mixture and to withdraw samples after various periods of incubation. It was while endeavouring to determine whether the inactivation process was an enzyme action that a better method was discovered. Yeast heated in saline at 100° for half to three-quarters of an hour proved a better agent than fresh yeast and a preparation of zymin powder similarly heated was the best inactivator found. Heated zymin was almost invariably successful in removing the third component from serum by 2 hours' treatment.

Before assuming that the process is one of adsorption several alternative possibilities must be eliminated:

(a) One of the enzymes of the yeast may destroy the third component. As already mentioned, prolonged heating of yeast and zymin at 100° improved their action so that an enzyme action appears unlikely. The possibility that the enzyme or enzymes concerned are intra-cellular and, therefore, more stable towards heat must, however, not be overlooked. In zymin powder the yeast although dead still retains some of its cellular structure.

(b) Inhibiting substances from the yeast may pass into the serum. Coca showed that this did not happen and our experience confirms his results. Further evidence against this theory was obtained by testing the action of yeast-treated sera on diminishing amounts of complement, when no inhibition of complement action was obtained.

(c) The yeast may raise the salt content of the serum so much that haemolysis is inhibited by osmotic action. This has been disproved in two ways. Yeast-inactivated serum requires exactly the same amount of distilled water to render it haemolytic for unsensitised red cells as does the corresponding normal guinea-pig serum, showing that the two sera are isotonic. Also, dialysis of yeast-treated serum against normal saline does not in any way affect its properties, *i.e.* its complete lack of complement activity and its activation by solutions containing the third component.

(d) The yeast may cause an acidity in the serum sufficient to precipitate the mid-piece or globulin fraction. As will be shown later this is not the case, since yeast-inactivated serum still contains mid-piece.

It seems certain therefore that yeast inactivates guinea-pig serum by combining physically or chemically with a heat-stable component of the serum.

It was thought possible that some other mechanical adsorbent might be substituted for yeast or zymin with the advantage that a more uniform reagent would be available. With this end in view, kaolin and charcoal, as typical adsorbents, were tried in place of yeast. Hecht [1923] states that kaolin adsorbs the mid-piece of complement most readily, but in our experiments both charcoal and kaolin when used in excess appear to remove all constituents of the serum and to have no specific effect on the third component. Kaolin

indeed seemed to remove the third component with least certainty, since several samples of serum treated with it, whilst quite inactive alone, contained some heat-stable factor and gave haemolysis when added to inactive yeast-treated serum. A curious fact noted with both kaolin and charcoal was that the serum after treatment was slightly alkaline in reaction whereas with yeast the reaction was distinctly acid.

In order to determine the relation of the third component to the other fractions of complement, serum was separated into globulin and albumin fractions or mid-piece and end-piece (hereafter known as *M* and *E*) by Liefmann's CO₂ method with various modifications described later. Two clear-cut fractions were almost always obtained which showed no complement action separately. Solutions *M* and *E* were tried for complement action in combination with inactive sera, produced by the action of various yeast and zymine preparations, and both solutions usually proved to be activating agents, proving the presence of the third component in each. It was found, however, that *E* had to be used, in nearly every case, in greater quantity to produce complete haemolysis and occasionally no third component could be demonstrated in this fraction. *M* and *E* were then heated at 56° until they contained no mid-piece or end-piece and practically the same result was obtained with the heated as with the unheated solutions.

It seems likely, therefore, that methods of protein separation have no specific action on the third component but that part of the latter is carried down with the globulin precipitate, the proportion varying with each individual serum and perhaps with the conditions of the experiment. In general, we have observed that in sera rich in heat-stable factor, both fractions contain this activating principle, and in cases where there is only a little, the globulin precipitate (*M*) appears to carry down the greater part, if not all.

Serum inactivated by yeast or zymine has been shown to contain both *M* and *E* but not the third relatively heat-stable component, and therefore separation of the inactive serum by CO₂ should give fractions free from the heat-stable factor. The separation was carried out and the mid-piece and end-piece fractions (*M*¹ and *E*¹) so obtained were found to have no complement action singly or when added together in varying proportions. *E*¹ could on every occasion be activated by small amounts of *M* from fresh serum, and *M*¹ was sometimes activated by *E*. The latter phenomenon only happened with sera which were shown otherwise to contain a relatively large amount of third component, part of which was present in the end-piece fraction. When no heat-stable factor was present in *E* no activation of *M*¹ by fresh end-piece was observed. These facts strongly support the theory that three factors are necessary for complement action, a globulin fraction (mid-piece), an albumin fraction (end-piece) and a third component or relatively heat-stable factor.

As a preliminary to further work on the third component we have attempted to activate yeast- and zymine-treated serum with aqueous and methyl alcoholic solutions of sodium oleate and egg-yolk lecithin and with solutions of egg

white. Up to the present the results are entirely negative in every case, *i.e.* the inactive solutions could not thus be rendered specifically haemolytic for sensitised red cells.

EXPERIMENTAL.

The haemolytic system. The experimental work was duplicated in laboratories at Leeds and Newcastle. In the former, ox cells and rabbit anti-ox serum constituted the haemolytic system, and in the latter, sheep cells and horse anti-sheep serum. In all cases guinea-pig serum was used as the source of complement.

The minimum haemolytic dose of complement varied, but for the sake of convenience the experimental details will be given from one laboratory. The solutions to be tested were pipetted into small 3" $\times$ $\frac{1}{2}$ " tubes, the bulk in each made the same (1 cc.) with 0.85 % saline, and 1 cc. of sensitised blood added. The results were read after 1 hour's incubation at 37° in an air incubator according to the following standards:

+	+	+	+	complete haemolysis	+	+	+	} intermediate degrees.
+	+			half-haemolysed	+			
— no haemolysis.								

Preparation of zymín and inactivation process. Zymín powder was prepared by digesting baker's yeast with equal parts of alcohol and ether, filtering and repeating the process. Finally, the treated yeast was washed with alcohol and dry ether, dried at room temperature and passed through a fine mesh sieve. Thus a fine impalpable yellowish white powder was obtained. It is interesting to note that none of the preparations we made by this method contained any zymase as judged by its action on glucose.

Zymín was added to 10 cc. of 0.85 % saline in a centrifuge tube until a thin paste was formed. The mixture was heated in a boiling water-bath for $\frac{1}{2}$ to 1 hour and then centrifuged, the supernatant saline being poured off. Fresh guinea-pig serum was then added to the zymín deposit and the whole stirred with a glass rod to make a viscid emulsion. The tube was incubated at 37° and samples withdrawn at 1½, 2, 3 and 4 hours. The serum was recovered from each sample by adding normal saline in an amount calculated to give 1/10 serum and centrifuging. Before using, the serum, which was definitely acid, was adjusted by means of a comparator to p_H 7.5. The amount of N/10 NaOH required to neutralise a volume equivalent to 5 cc. of the original serum was in the neighbourhood of 1.0 cc. Inactivation by unheated zymín and by yeast and heated yeast was carried out in a similar way. A curious fact occasionally noticed was that treatment with a greater quantity of yeast for the same time or for a longer period failed to inactivate the serum. No explanation of this phenomenon has yet been found.

The following table is typical of the results which have been repeatedly obtained:

Table I.

0.5 cc. 1/10 complement	+++
0.5 cc. H_{20} (1/10 complement heated 20 minutes at 56°)	-
0.5 cc. H_{40} (1/10 complement heated 40 minutes at 56°)	-
0.5 cc. Z_1 (1/10 zymin-inactivated complement. 2 hrs.)	-
0.5 cc. Z_2 (1/10 zymin-inactivated complement. 3 hrs.)	-
0.5 cc. Z_1 + 0.5 cc. H_{20}	+++
0.5 cc. Z_2 + 0.5 cc. H_{20}	+++
0.5 cc. Z_1 + 0.5 cc. H_{40}	+++
0.5 cc. Z_2 + 0.5 cc. H_{40}	+++

Similar results were obtained with zymin and yeast which had previously been heated in the dry state at 100° in an air oven for 1 hour and which were then washed with normal saline.

Preparation of mid-piece and end-piece and their effect upon complement inactivated by zymin or yeast. Complement was diluted 10 times with distilled water, cooled in ice and saturated with CO_2 by passing the gas for 10–15 minutes. The globulin separated as a flocculent precipitate and was removed by centrifuging. The supernatant fluid containing end-piece (E) was decanted into a flask, sufficient 10 % saline added to render it isotonic with normal saline, and then the flask was evacuated twice to remove CO_2 . The mid-piece (M) precipitate was lightly washed twice with distilled water, and then dissolved in a volume of normal saline equal to that of the end-piece. Both solutions were finally adjusted in a comparator to p_H 7.5. E was always quite acid (about p_H 6); M , although slightly acid, was unbuffered. The importance of removing the CO_2 and adjusting these solutions seems to have been overlooked by many workers on complement. Excess of CO_2 has a marked anti-complementary action and although in most of our solutions the acidity was not sufficient to affect the results, it is obviously desirable that all solutions should approximate in reaction to normal serum. Evacuation of E removes most of the CO_2 and for ordinary purposes this treatment is perhaps all that is necessary.

Table II.

$\left. \begin{matrix} M_{15} \text{ and } M_{30} \\ E_{15} \text{ and } E_{30} \end{matrix} \right\} = E \text{ and } M \text{ heated at } 56^\circ \text{ for 15 and 30 minutes.}$			
0.2 cc. 1/10 complement	+++	0.5 cc. E	-
0.5 cc. 1/10 complement	+++	1.0 cc. E	-
0.5 cc. M	-	0.5 cc. M + 0.5 cc. E	+++
1.0 cc. M	-	0.2 cc. M + 0.2 cc. E	+++
0.5 cc. Z_1 + 0.5 cc. H_{20}	+++	0.5 cc. Z_1	-
0.5 cc. Z_1 + 0.5 cc. H_{40}	+++	0.5 cc. Z_1 + 0.5 cc. M	+++
0.5 cc. H_{20} + 0.5 cc. E ...	-	0.5 cc. Z_1 + 1.0 cc. M	+++
0.5 cc. H_{20} + 1.0 cc. E ...	+	0.5 cc. Z_1 + 0.5 cc. E	+
0.5 cc. H_{20} + 0.5 cc. M ...	-	0.5 cc. Z_1 + 1.0 cc. E	+++
0.5 cc. H_{20} + 1.0 cc. M ...	-	0.5 cc. Z_1 + 0.5 cc. M_{15}	+++
0.5 cc. H_{40} + 0.5 cc. E ...	-	0.5 cc. Z_1 + 1.0 cc. M_{15}	+++
0.5 cc. H_{40} + 1.0 cc. E ...	-	0.5 cc. Z_1 + 0.5 cc. E_{15}	-
0.5 cc. H_{40} + 0.5 cc. M ...	-	0.5 cc. Z_1 + 1.0 cc. E_{15}	+
0.5 cc. H_{40} + 1.0 cc. M ...	-	0.5 cc. Z_1 + 0.5 cc. M_{30}	+++
		0.5 cc. Z_1 + 1.0 cc. M_{30}	+++
		0.5 cc. Z_1 + 0.5 cc. E_{30}	-
		0.5 cc. Z_1 + 1.0 cc. E_{30}	+

Effect of excess of CO₂ on complement action. Michaelis and Skwirsky [1910, 1] have shown that the action of complement varies with the reaction of the solution. By the aid of phosphate buffer mixtures these authors found that an acid reaction inhibits complement action and that the reaction proceeds best in neutral or faintly alkaline solutions. In view of this we have investigated the effect of CO₂ on the action of decreasing amounts of guinea-pig complement. Normal saline was saturated with CO₂ and this solution added to varying strengths of diluted normal guinea-pig serum.

Table III.

0.5 cc. 1/10 normal complement				+	+	+	+
0.5 cc. " "			+ 0.5 cc. CO ₂ -saturated saline	+	+	+	+
0.5 cc. " "			+ 1.0 cc. " "	+	+	+	+
0.5 cc. 1/20 normal complement				+	+	+	+
0.5 cc. " "			+ 0.5 cc. " "	+	+	+	+
0.5 cc. " "			+ 1.0 cc. " "	+	+		
0.5 cc. 1/30 normal complement				+	+	+	+
0.5 cc. " "			+ 0.5 cc. " "	+	+		
0.5 cc. " "			+ 1.0 cc. " "				
0.5 cc. 1/40 normal complement				+	+	+	+
0.5 cc. " "			+ 0.5 cc. " "				
0.5 cc. " "			+ 1.0 cc. " "				

Separation of zymin-inactivated serum into mid-piece and end-piece fractions. Zymin-inactivated guinea-pig serum was centrifuged, the supernatant liquid diluted with nine times its volume of distilled water and a slow stream of CO₂ passed through for 10–15 minutes. The two fractions were separated and dealt with as described for normal serum, the solutions being adjusted to p_H 7.5.

Table IV.

M and E =normal mid-piece and end-piece fractions.

M^1 and E^1 =mid- and end-piece from zymin-inactivated serum (by CO₂ separation).

M_{15} , M_{30} , E_{15} and E_{30} as in Table II.

M^1 and E^1 separately inactive in quantities of 0.5 cc. and 1.0 cc.

0.5 cc. M^1 + 0.5 cc. E^1	...	...	...	—
0.5 cc. M^1 + 0.5 cc. E ...	...	...	...	+
0.5 cc. M^1 + 0.5 cc. E_{15}	...	...	...	—
0.5 cc. E^1 + 0.5 cc. M	...	...	...	+++
0.5 cc. E^1 + 0.5 cc. M_{15}	...	...	...	—
0.5 cc. M^1 + 0.5 cc. E^1 + 0.5 cc. normal saline	...	...	...	—
0.5 cc. M^1 + 0.5 cc. E^1 + 0.5 cc. M	...	...	...	+++
0.5 cc. M^1 + 0.5 cc. E^1 + 0.5 cc. M_{15}	...	...	...	+++
0.5 cc. M^1 + 0.5 cc. E^1 + 0.5 cc. M_{30}	...	...	...	+++
0.5 cc. M^1 + 0.5 cc. E^1 + 0.5 cc. E	...	...	...	+
0.5 cc. M^1 + 0.5 cc. E^1 + 0.5 cc. E_{15}	...	...	...	—
0.5 cc. M^1 + 0.5 cc. E^1 + 0.5 cc. E_{30}	...	...	...	—

These results were obtained with a serum which did not contain a large amount of heat-stable factor and little was to be found in the normal end-piece fraction. Thus the zymin-inactivated serum contains the globulin and albumin fractions but no third component.

SUMMARY.

(1) The inactivation of guinea-pig serum by the action of yeast can also be effected by a zymin preparation. The serum so obtained can have its complement activity renewed by the addition of serum inactivated by heating at 56°.

(2) The possibility of explanations other than that already offered—viz. the removal of a “third component” or relatively heat-stable factor—has been investigated and disproved.

(3) Yeast and zymin heated in normal saline or in the dry condition at 100° for $\frac{1}{2}$ to 1 hour are more efficient inactivators than the unheated substances, and thus the inactivation process does not appear to be one of enzyme action.

(4) Kaolin and charcoal are not similar to yeast in their action on serum.

(5) Zymin, like yeast, removes a heat-stable component of complement which is usually present in both mid-piece and end-piece as obtained by Liefmann's CO₂ method. The greater bulk of the heat-stable component is usually present, however, in the mid-piece and in some sera none can be found in the end-piece. No definite chemical or physical relationship appears to exist between this third component and the mid-piece and the end-piece.

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