

REACTIVE LYSIS: THE COMPLEMENT-MEDIATED LYSIS OF UNSENSITIZED CELLS

II. THE CHARACTERIZATION OF ACTIVATED REACTOR AS $\overline{C56}$ AND THE PARTICIPATION OF C8 AND C9*

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Reactive lysis is the consequence of the interaction of two reagents. One, previously called indicator, is a normal serum protein and has been identified as C7 (1); the other is generated by complement activation from certain sera (referred to as "reactor" sera) and has been called activated reactor. In this paper the nature of the activated reactor is explored and the course of the reaction analyzed.

Materials and Methods

These are described in the preceding paper (1). Diisopropyl fluorophosphate (DFP) was obtained from Boots Pure Drug Co., Nottingham, England.

1:10 phenanthroline hydrate was obtained from Hopkins & Williams, Ltd., Chadwell Heath, Essex, England. A 0.5 M solution was made in dimethylformamide and this was diluted 1:50 in the E/EDTA/agarose mixture.¹

Quantitation of Activated Reactor.—An estimate of activated reactor was obtained by filling a standard size well in an E/EDTA/agarose plate with activated reactor and allowing diffusion to occur for 18 hr at room temperature. The plate was then immersed in 1:50 guinea pig EDTA complement and incubated at 37°C for 6 hr. The diameter of the zone of lysis was measured.

RESULTS

Fractionation of Activated Reactor

Absorption with Zymosan (4 mg/ml) and Precipitation as Euglobulin.—

These were performed in the usual way. Reactor activity in the euglobulin fraction, when redissolved in 0.3 N NaCl, pH 7.2, was stable on storage both at 4°C and at -20°C. Addition of glycerol to a final concentration of 25% allows the activated reactor euglobulin to be stored, certainly for some months, at -20°C without loss of activity.

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¹ 1% agarose plates made up in 0.01 M ethylenediaminetetraacetic acid (EDTA) in buffered saline and containing 1% sheep red cells (1).

The pseudoglobulin fraction was devoid of reactor activity, but contained an inhibitor of reactor hemolysis. For this reason, the euglobulin was a much better source of activated reactor than whole activated serum.

Fractionation on Diethylaminoethyl (DEAE)-Cellulose.—

The activated reactor euglobulin was fractionated on DEAE-cellulose in 40% glycerol containing buffers essentially as described for the fractionation of C7 in (1).

The starting buffer was 0.01 M phosphate, pH 7.9, and gradient elution was performed with 0.3 M NaCl in starting buffer.

Activated reactor factor (activated R) activity was eluted at a conductivity of 2.8–4.0 millimhos/cm which in the presence of 40% glycerol, corresponds approximately to 0.20 N NaCl. The active fractions were pooled and concentrated by positive pressure ultrafiltration in a Diaflo cell.

Antigenically, the concentrated material was markedly heterogeneous. It contained the activities of C9 and C1 as well as of activated R.

*Fractionation on Sephadex G-200.—*The DEAE-purified activated R was further fractionated on Sephadex G-200. The elution diagram is shown in Fig. 1. Activated R activity was found in the fractions immediately following the exclusion peak.

The activated R from the Sephadex column had a protein concentration of 220 μ g/ml. and was purified about 300-fold, compared to the activated R euglobulin. Because of the limited amount of material available, further purification was not attempted.

The preparation was still contaminated with C1 as shown by its inactivation of C4. This activity was removed by treatment with 10^{-3} M DFP which had no effect on activated R activity. The DFP-treated preparation was used as "functionally-purified" activated R.

On examination in Ouchterlony plates, the functionally pure activated R gave several lines with a highly polyvalent anti-human globulin serum. One of these gave a line of identity with anti-C3 (Fig. 2) and another was shown separately to give a line of identity with anti-IgG. Precipitin lines with anti C5 and with anti-C6 were faint. However, it may be seen in Fig. 2 that the lines given by anti-C5 and anti-C6 fuse, indicating that these two components were complexed in activated R.

*Molecular Weight of Activated Reactor.—*The sedimentation coefficient of activated R was measured by sucrose gradient ultracentrifugation (Fig. 3) and was found to be 11.7 S (mean of three values).

The diffusion coefficient was measured by two techniques:

Firstly, the L-plate method of Allison and Humphrey (2) was used. Activated R was diffused against C7 in E/EDTA/agarose plates. After 48 hr, the plate was immersed in guinea pig EDTA-C to develop the hemolytic line. This line formed at an angle of 39° with the trough containing activated R. Since the diffusion coefficient of C7 is known to be 4.0 (1) the diffusion coefficient of activated R could be calculated to be 2.6×10^{-7} cm²/sec.

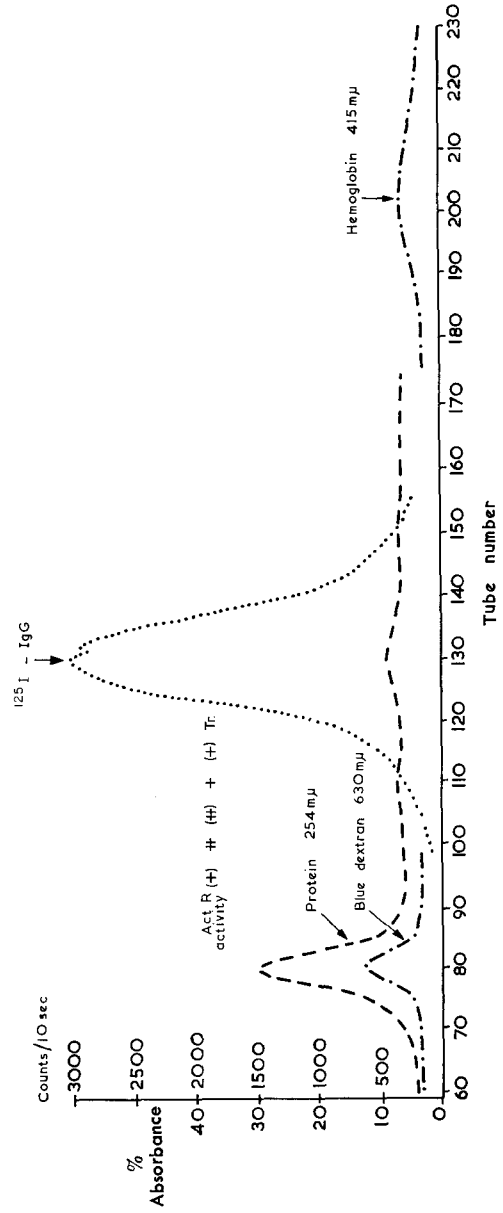


FIG. 1. The fractionation of activated R on Sephadex G-200. The DEAE-fractionated activated R was applied to a 5 X 100 cm Sephadex G-200 column equilibrated with 0.1 M Tris buffer, pH 7.2, containing 0.5 M NaCl 0.01 M Na₂S₂O₈ and 0.01 M EDTA. The column was pumped upwards at 26 ml/hr and 6.5 ml fractions were collected and tested for activated R. The markers were run on the same column under identical conditions but as a separate fractionation.

Secondly, a value for the diffusion coefficient was calculated from the Sephadex G-200 fractionation by plotting the elution volume against $1/D$ (3). Taking a value of 6.8 for the diffusion coefficient of hemoglobin (peak at tube 204) and of 3.8 for IgG (peak at tube 130) the diffusion coefficient of the exclusion peak (peak at tube 80) is equal to or less than 2.9 and the diffusion peak of activated R (peak at tube 100) is 3.2.

Taking the measured values of 11.7 for $s_{20,w}$ and the mean value of 2.9 for $d_{20,w}$, and assuming a value of 0.73 for the partial specific volume (\bar{v}), the molecular weight was calculated by the Svedberg equation to be in the region of 360,000 and the frictional ratio was calculated by Oncley's equations to be in the region of 1.6.

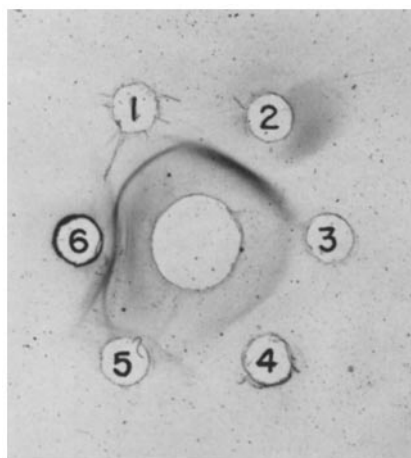


FIG. 2. The antigenic analysis of functionally purified activated R. The center well contains functionally purified Activated R. The numbered wells contain: 1. Anti-C3 [F(ab')₂ fraction]; 2. Polyvalent anti-human globulin serum 1; 3. Anti C5 (IgG fraction). (This fraction contains some human serum protein); 4. Anti C6 IgG; 5. Anti C3 [F(ab')₂ fraction]; 6. Polyvalent anti-human globulin serum 2.

Antigenic Identification of Activated Reactor by Inhibition of Reactive Hemolysis.—Specific antisera were tested for their capacity to inhibit reactive hemolysis. IgG fractions of antiserum or F(ab')₂ fractions of purified antibody² were used to avoid nonspecific inhibition.

The following patterns of reactivity were observed:

No inhibition (Fig. 4a) where the hemolytic lines showed no interruption. This was found with antibody to immunoglobulins G, A, M, and D; to C4 and to C3; and to properdin.

Inhibition of indicator (Fig. 4b) where the hemolytic lines were deviated

² Lachmann, P. J. The purification of specific antibody F(ab')₂ by the pepsin digestion of antigen-antibody precipitates. Manuscript submitted for publication.

and inhibited by a reaction between antibody and indicator. This pattern was shown only by antibody to indicator (C7) (1).

Inhibition of activated reactor (Fig. 4c) where the hemolytic lines were deviated and inhibited by a reaction of antibody with reactor.

This pattern was always given by anti-C5 and anti-C6. Anti-C8 also gave this pattern when an activated R euglobulin was used with purified C7 as source of indicator. However, when purified activated R was used and the hemolytic lines developed with guinea pig EDTA-complement (see below) anti-C8 was not inhibitory.

This analysis indicates that activated R contains C5 and C6. The molecular

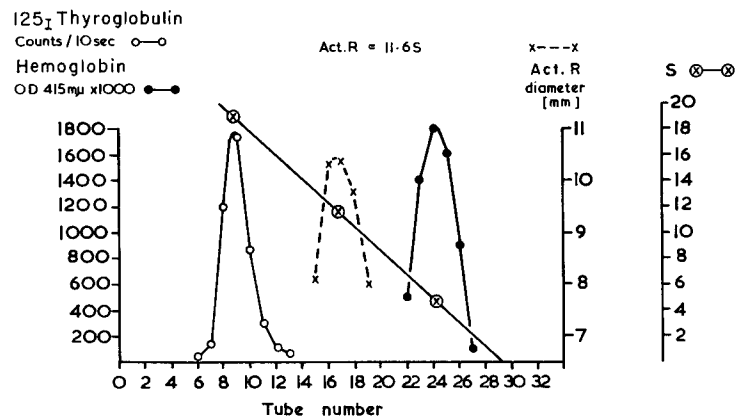


FIG. 3. The ultracentrifugal analysis of activated R. A 4.8 ml linear sucrose gradient from 8–36% sucrose was used. 0.2 ml activated R containing the markers was applied. Centrifugation was performed in a Spinco SW39 rotor for 20 hr at 35,000 rpm. 10-drop fractions were collected from the bottom of the tube and tested as shown.

weight of C6 is around 150,000 (4); that of C5, with a sedimentation coefficient of 8.7 S is likely to be above 200,000. Activated R with a molecular weight in the region of 360,000 can therefore contain only one molecule of each. The data is insufficiently accurate to exclude the presence of another much smaller molecule in the complex.

The Role of C8 and C9 in Reactive Lysis.—When functionally purified preparations of activated R and C7 were tested in red cell containing plates, lines of lysis were not seen after interaction but could be developed by immersing the plates briefly in dilute guinea pig EDTA-C. Prolonged incubation in EDTA-C gave zones of lysis wherever activated R was present in the plate and may be used as a method for quantitating activated R.

Table I shows the hemolytic lines obtained using various indicator and activated reactor reagents.

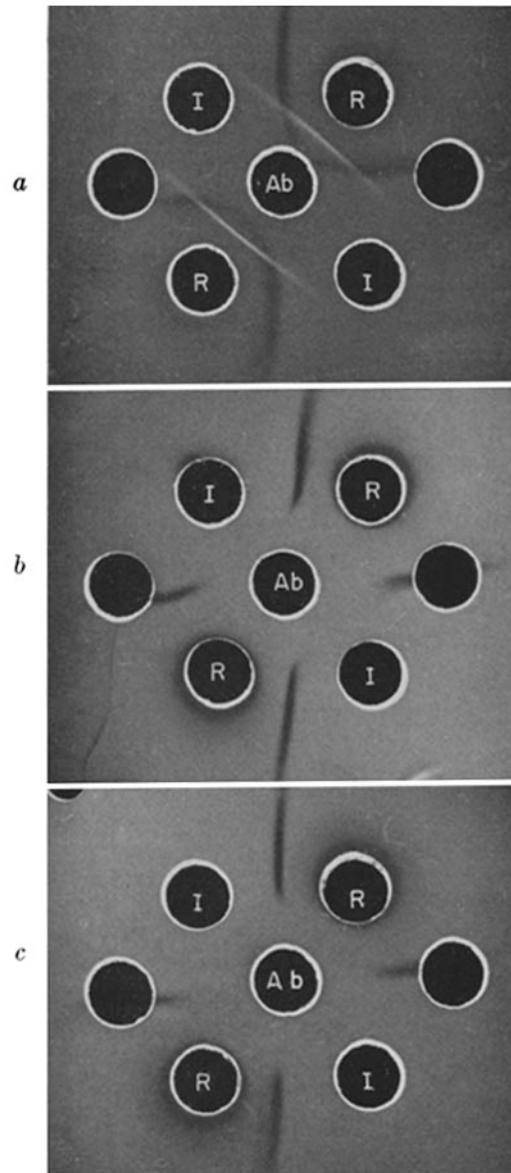


FIG. 4. The antigenic analysis of reactive lysis by inhibition of hemolysis by antisera. (a) shows no inhibition. The inhibiting antibody is anti-C3 which gives a line of precipitation with activated R. (b) shows inhibition of indicator. The inhibiting antibody is anti-C7. (c) shows inhibition of activated R. The inhibiting antibody is anti-C6. R, activated R. 7, C7. Ab, IgG fraction of antiserum. The unlabeled wells contained saline.

It can be seen that both C8 and C9 are needed for hemolysis to occur and that it is immaterial in which reagent they are present. The need for C9 is abolished if 0.01 M phenanthroline is incorporated in the plate (5).

It is concluded that the primary product of the interaction of activated R and C7 in the presence of red cells (E) is EC567.

Reactive Hemolysis in the Test Tube.—Although it is convenient to carry out reactive hemolysis in immunodiffusion plates, the reaction works equally well in the test tube.

TABLE I
Hemolytic Lines Obtained Using Various Indicator and Activated Reactor Agents

Source of activated R	Source of C7	Hemolytic lines		
		After diffusion in		Developed by EDTA-C (C7, C8, C9)
		Normal plate	Phenanthroline plate	
Activated R euglobulin (C56, C8, C9)	Functionally pure (C7)	+	—	—
DEAE fraction (C56, C9)	Functionally pure (C7)	0	0	+
DEAE fraction (C56, C9)	DEAE fraction (C7, C8)	+	—	—
Functionally pure (C56)	Normal euglobulin (C7, C8, C9)	+	—	—
Functionally pure (C56)	DEAE fraction (C7, C8)	0	+	+
Functionally pure (C56)	Functionally pure (C7)	0	0	+
Functionally pure (C56)	None	0	0	0*

* Produces circular zones of hemolysis after incubation for several hours.

Late acting complement components present in the various reagents are shown in parenthesis. Diffusion between activated R and C7 takes place overnight at room temperature. Phenanthroline plates contain a final concentration of 0.01 M phenanthroline. Plates are immersed in 1:50 guinea pig serum in 0.01 M EDTA for 15 min at 37°C to develop hemolytic lines. +, hemolytic line present; 0, no hemolytic line; —, not done.

When E was mixed with functionally purified activated R and an equivalent amount of C7 then added, no lysis occurred but the cells acquired the capacity to be lysed by guinea pig EDTA-C. This activity is "heat stable" as shown in Fig. 5b and corresponds to the hemolytic activity of EC567.

Although the EC567 cells are so stable once they are formed, the capacity of the product of activated reactor and C7 interaction to form these cells is very short lived having a half life of 1 min or less (Fig. 5a).

Titration of the functionally purified C7 and activated reactor were performed in the presence of excess of the other reagent to determine the least amounts necessary to produce EC567. The C7 preparation with a protein concentration of 280 µg/ml had a titer of 600, i.e., about 45 µµg were needed to

give 50% hemolysis of about $10^7 E$. The activated reactor with a protein concentration of $220 \mu\text{g/ml}$ had a titer of 170, i.e., $130/\mu\text{g}$ were needed to give 50% hemolysis of about $10^7 E$. For both reactants, this works out in the region

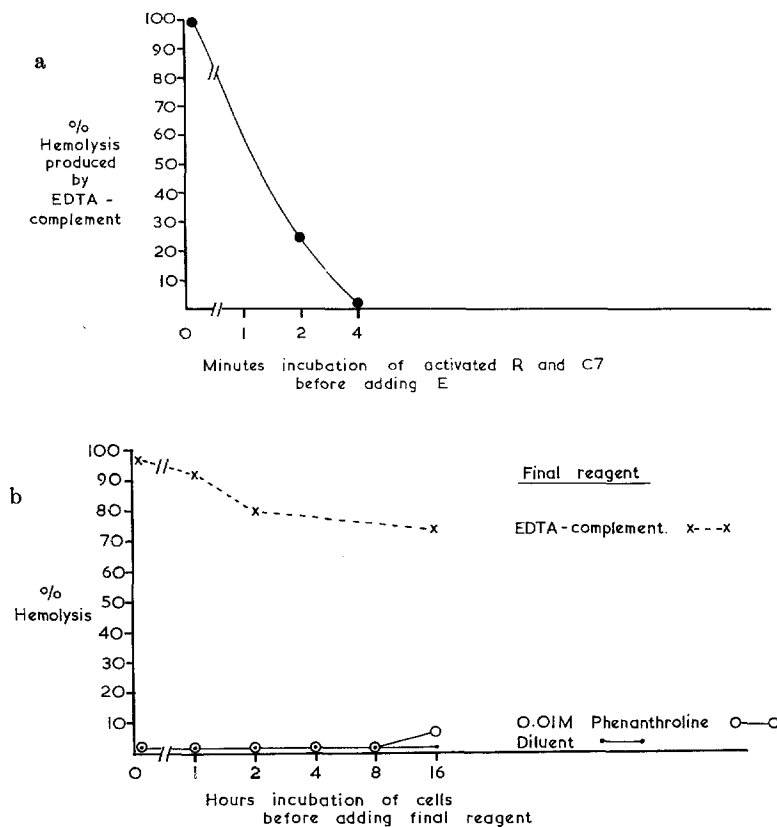


FIG. 5. The heat stability of C567 for making EC567 and of EC567 for lysis by EDTA-C (a) Activated R and C7 were mixed at 37°C . After the incubation period E was added and incubation continued for 15 min at 37°C , when guinea pig EDTA-C was added. Lysis was measured after a further 30 min at 37°C . (b) EC567 were incubated at 37°C . After the incubation period, the cells were suspended in guinea pig EDTA-C, 0.01 M phenanthroline, or in diluent alone and incubated for 30 min more at 37°C before measuring lysis.

of 2×10^4 molecules/red cell, assuming the preparation to be pure. Since, however, on a protein basis, some of the indicator preparation is IgG and much of the activated reactor is β_{1A} -globulin, this figure is a considerable overestimate. There was no inhibition of lysis by excess of either reagent.

Antigenic Analysis of EC567 made with Activated R and C7.—EC567 made with 10 times the 50% hemolytic endpoint were analyzed antigenically by the

use of specific antibodies to complement components. The results are shown in Table II. It is seen that C5 is the main agglutinin on these cells and anti-C5 was the only antiserum to give agglutination strong enough to be read by the resuspension technique. Anti-C6 and anti-C7 also agglutinated the cells, whereas anti-C4 and anti-C3 quite failed to do so. The absence of C3 on the cells was confirmed by their failure to react in immune adherence or with conglutinin in the presence of conglutinin-activating factor (KAF). Two serum

TABLE II
Characterization of Reactive Hemolysis Cells as EC567 by the Antiglobulin Reaction

Agglutinating serum	Indicator Titer of agglu- tinating serum	Reactive hemolysis cells*					E	Control† cells
		25	125	625	3125	15,625		5
Anti-C4 IgG		0	0	0	0	0	0	0
Anti-C3 IgG		0	0	0	0	0	0	0
Anti-C5 IgG		++	++	++	++	+	0	0
Anti-C6 IgG		++	++	0	0	0	0	0
Anti-C7 IgG		++	++	+	0	0	0	0
Anti-C8 IgG		0	0	0	0	0	0	0
Anti-properdin IgG		0	0	0	0	0	0	0
	<i>Titer on EAC rabbit:</i>							
Human salivary IK	>2000	+	+	0	0	0	0	0
Rabbit serum IK [R150]	>2000	0	0	0	0	0	0	0
Rabbit serum IK 1197/ 6/6	>2000	0	0	0	0	0	0	0
	<i>Reaction with EAC rabbit:</i>							
	++++	10 doses conglutinin				0	0	0
	++++	10 doses conglutinin + KAF				0	0	0
	++++	Immune adherence				0	0	0

* 1 ml purified DFP-treated activated R, + 1 ml 10% E + 0.2 ml. C7/5 min, 37°C, washed well.

† 1 ml purified DFP-treated activated R, +0.2 ml C7/5 min 37°C/1 ml. 10% E/5 mins. 37°C, washed well.

immunoconglutinins also failed to agglutinate the cells but immunoconglutinins (IK) from human saliva (6) did agglutinate them to a low titer, indicating that autoantibodies to the 567 determinants can be formed.

The Interaction between Activated Reactor and C7 in the Absence of Cells.—When activated R and C7 interact in solution, the hemolytic activity of their product was rapidly lost (Fig. 5a). However, this hemolytically inactive C567 still inactivated C8 and the capacity to do so was quite stable (Table III).

If C8 was present, the resulting C5678 inactivated C9. Thus the DEAE fraction of activated R may be freed of C9 by the addition of one-fifth of its equivalent amount of the DEAE fraction of C7, which also contains C8, but

not by the same amount of C7 alone. Similarly, if just sufficient activated R was added to normal human serum to destroy its hemolytic activity on EA, the resulting reagent was depleted of C9 and with EA gave EAC 1 ~ 8. If enough activated R to deplete all the C7 was used, the resulting reagent gave rise to EAC 1 ~ 3.

TABLE III
The Consumption of C8 by Activated R-C7 Complex

Added to titration of C8	Dilution of C8 Preparation					
	33	100	300	900	2700	Diluent
C7	++	++	++	+	(+)	0
Activated R; mix; then C7	++	0	0	0	0	0
Activated R + C7 preincubated 5 min at 37°C*	++	(+)	0	0	0	0

* Incubation of the activated R-C7 (C567) complex for 1 hr at 37°C or overnight at 4°C have been found not to affect its capacity to inactivate C8.

C7: approximately 2 μ g of the most highly purified material was used. Activated R: DEAE-fractionated activated R was used. The amount was one-fifth of the amount equivalent to 2 μ g C7. All mixtures therefore contained ample free C7. The final mixtures were allowed to diffuse in E/EDTA/agarose plate, incorporating 0.01 M phenanthroline against a functionally purified activated R preparation.

The scoring describes lines of reactive hemolysis. ++, line of complete hemolysis. +, obvious line of partial hemolysis. (+), weak line of partial hemolysis.

TABLE IV

Source of serum	Lines of reactive lysis given by sera treated with		
	Saline	Anti- α_2 macroglobulin IgG	Anti C7 IgG
Normal (nonreactor)	0	0	+
Acute phase (reactor)	+	+	++

0.5 ml of serum was treated with 0.1 ml IgG preparation for 30 min at 37°C and centrifuged. The supernatants with Ca and Mg restored were tested for reactor activity in plates by diffusion against a C7 preparation.

The Properties Conferring Reactor Activity on a Serum.—The capacity to generate activated R has been found in a minority of human sera—generally those in the “acute phase” of inflammation (9) and the factors that confer reactor activity on these sera are not known.

It seems quite likely that the balance between the amount of C56 that can be generated and the amount of C7 that is available to react with it determines whether a serum (after zymosan absorption) contains activated R or C7 or neither.

Evidence in support of this view has been obtained by showing that non-

reactor serum treated with anti-C7 IgG acquired reactor activity (Table IV).

Further evidence for this view has been obtained by studying C7 levels in a hospital population and comparing these with the incidence of reactor sera. The results shown in Fig. 6 demonstrate that the incidence of reactor sera is highest at the lowest C7 levels and falls steadily with increasing amounts of C7.

Finally preliminary studies carried out using reagents kindly made available by Dr. S. Ruddy, have shown that activated R could be generated by the

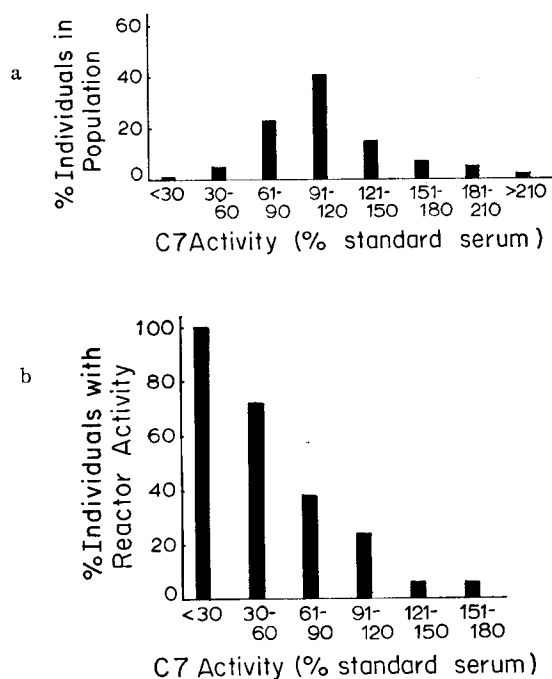


FIG. 6. (a) The serum concentration of indicator (C7) in 250 medical and surgical hospital patients. (b) Incidence of reactor activity in different indicator ranges. C7 levels were measured as described in (1).

interaction of partially purified preparations of C5 and C6, with EAC14^{oxy}23, but not with EAC14^{oxy}2.

The possibility that properdin which occurs in greater quantity in acute phase sera might be involved in the generation of reactor was also considered. However antiserum to properdin, kindly supplied by Dr. G. Naff, was without inhibitory effect on reactive hemolysis and did not agglutinate EC567 made with activated R and C7. If therefore properdin plays any part in reactor activity it does so without being incorporated into activated R.

DISCUSSION

Fig. 7 shows schematically the process of reactive hemolysis as it emerges from the experiments that have been described.

Complement activation in certain sera leads to the generation of activated R ($\overline{C56}$), a stable complex involving the fifth and sixth components of complement in 1:1 combination. This is always associated with loss of C7 and is believed to reflect a relative excess in these sera of $\overline{C56}$ over C7. In support of this view

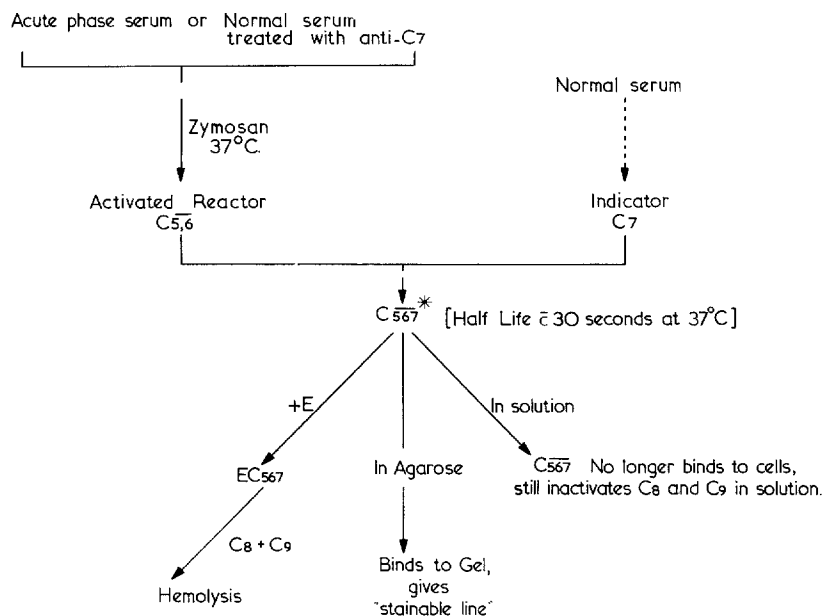


FIG. 7. Schematic representation of reactive lysis. The bar on $\overline{C56}$ and $\overline{C567}$ refers to activation of the site that allows them to react with subsequent component. The asterisk $\overline{C567}^*$ refers to activation of the binding site.

are the observations: (a) that depleting C7 in normal sera with anti-C7 causes them to behave as reactor sera; (b) that reactor sera usually have low levels of C7; and (c) that treating C5 and C6 with EAC1423 gives rise to activated R.

It remains unclear why some complement activating systems, e.g., zymosan and its natural antibody, or lightly sensitized *Proteus* should be so much better at generating activated R than, for example, aggregated gamma globulin (9), but the factors involved are presumably the same that cause these polysaccharide systems to be so good at making reagents mainly depleted in late complement components.

The interaction of $\overline{C56}$ with C7 gives rise to a complex which has for a brief

period the capacity to attach to erythrocyte and, according to preliminary evidence, to nucleated cell membranes; to bacteria; to other hydrophobic surfaces including lipid emulsions; and to agarose gel where the binding can be seen as a "stainable line". The fixed complex confers on cells the hemolytic activity associated with the "heat stable" complement intermediate EAC 1 ~ 7. The lysis produced by reactive hemolysis has been shown in association with Dr. R. R. Dourmashkin to show the same electron microscopic lesions as are found in normal immune hemolysis. The heat stable cells made by reactive hemolysis, however, have neither antibody nor the earlier complement components bound on them. They are thus negative in immune adherence. This emphasises that the hemolytic process requires the complement activation as far as the C3 stage, only to generate C56 which can then provoke lysis in conjunction with the later components.

Immune hemolysis of cells bearing only complement components after the C5 stage has also been produced by a somewhat different method by Götze and Müller-Eberhard (10).

If the activated C567 complex fails to bind to membrane, it rapidly loses the capacity to do so and appears in solution as a stable complex that remains able to inactivate C8 and C9 in solution. Experiments to be published elsewhere have shown that this binding site inactivated C567 complex has chemotactic activity for neutrophils and thus corresponds to the C567 chemotactic factor generated in the normal complement fixation reaction (11).³

The evanescent activation of the binding site of C567 and the simultaneous long-lasting activation of its hemolytic site is very similar to the situation described for C4 by Müller-Eberhard and his colleagues (12).

It would seem highly probable that the reaction of C5, C6, and C7 in the normal complement sequence follows basically the same pattern as in reactive hemolysis; the generation of C56 (in this case, presumably held loosely at the complement fixation site), the interaction with C7 and subsequent firm binding to the cell membrane and the acquisition of the heat stable hemolytic property. This mechanism would be intermediate between the "sequential action" and "functional unit" mechanisms of C5, C6, and C7 action discussed by Nilsson and Müller-Eberhard (13) and would explain why the intermediates between EAC 1 ~ 3 and EAC 1 ~ 7 are unstable.

One difference between the two situations however lies in the amount of C567 bound to cells. In the normal complement sequence, the majority of C567 escapes into solution, and the amount of C5 bound (0.5% of input) is too small to be detected antigenically with an anti-C5 serum (13, 14). EC567 made with activated R and C7 are on the other hand very strongly agglutinated by anti-C5. The difference may reflect the much greater area of membrane

³ Lachmann, P. J., A. B. Kay, and R. A. Thompson. Manuscript in preparation.

available if $\overline{C56}$ is generated in solution and not at the complement fixation site, or it may be that the coating of the cell with C3 limits the uptake of $\overline{C567}$.

The significance of reactive hemolysis *in vivo* is not known. It provides a mechanism by which complement activation at one site can lyse normal cells at a distance, but the extent to which reactive hemolysis as a mediator of "immune complex" damage has still to be evaluated. It would however seem quite likely that the lysis of paroxysmal nocturnal hemoglobinuria (PNH) cells is due to reactive hemolysis. PNH cells have been found in our hands to be extremely sensitive to reactive hemolysis and there is already evidence (15) that fluid phase complement activation is associated with PNH cell lysis.

Whatever its role *in vivo*, *in vitro* the phenomenon provides a useful tool for the study of the late stages of complement action. It provides the basis for reproducible test systems for the late-acting complement components, and inhibitors and antibodies to them, without the need for extensively purified preparations of all nine components. For example it has already been shown using these cells that there is an agglutinogen in EC567 recognized by human salivary I-K's so that this is a further complement intermediate that is autoantigenic.

SUMMARY

It has been shown that the "activated reactor" that is produced in certain human sera by complement activation is a stable complex of the fifth and sixth component of complement ($\overline{C56}$). On interaction with C7, the indicator factor, a complex $\overline{C567}$ is formed which for a short time (half-life less than 1 min) has an activated binding site and can attach itself to normal red cell membranes, conferring on them the hemolytic properties of the "heat stable" complement intermediate EC 1 ~ 7, the capacity to be lysed by C8 and C9. These cells have neither antibody nor the complement components up to C3 bound on them.

The binding site—activated $\overline{C567c}$ —can similarly bind to other hydrophobic surfaces, including agarose gel where it forms a "stainable line".

If the complex is not bound to a surface, the binding site decays and the resulting complex will no longer give rise to lysis. However it will still inactivate C8 and C9 in solution. The sera that can generate activated reactor apparently do so because they have an excess of C5 and C6, compared to their content of C7.

The phenomenon of reactive lysis thus represents complement-mediated lysis of unsensitized cells initiated at the C5 stage by a stable complex ($\overline{C56}$) which was generated by complement activation at a distance.

The immunochemistry of the phenomenon is described and some of its implications discussed.

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