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REACTIVE LYSIS: THE COMPLEMENT-MEDIATED LYSIS OF UNSENSITIZED CELLS

I. THE CHARACTERIZATION OF THE INDICATOR FACTOR AND ITS IDENTIFICATION AS C7*

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"Reactive hemolysis" describes a form of red cell lysis which can be differentiated from classical complement lysis by the fact that it occurs in the absence of antibody on the red cells and in the presence of ethylenediaminetetraacetic acid (EDTA) (1). The salient feature is that the hemolysis occurs as a result of the fluid phase interaction of two serum factors in the presence of the cells. These factors have provisionally been called activated reactor, and indicator (hereafter frequently referred to for convenience, as activated R and I, respectively). I is a normal serum protein whereas activated R can be obtained only from some sera (usually acute phase sera), and has to be generated by incubation at 37°C with such agents as sensitized bacteria or zymosan, in circumstances which allow complement activation. The evidence that activated R is a stable complex of the complement components C5 and C6 is given in a separate publication (2).

During the generation of activated R in those sera which show it, I activity is lost as a consequence of the mutual inactivation of the two factors, the resulting serum showing the activity of the factor present in excess. Evidence is presented here that I is identical with the seventh component of complement (C7). Its physicochemical and antigenic characteristics and a convenient method of quantitation are described.

Materials and Methods

Activated Reactor was prepared as described in (2).

Antisera.—Antisera specific to human IgG, IgA, IgM, and IgD were obtained as previously described (1). Polyvalent anti-human serum was obtained from Burroughs & Wellcome Co., Beckenham, England.

Antisera to Complement Components.—

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C3: antiserum to C3 was made by immunizing rabbits with immune precipitates coated with human complement. A further antiserum was made by injecting rabbits with well washed precipitin lines from immunodiffusion plates between the antiserum and human C3. This second antiserum was monospecific when tested against a variety of serum fractions by Ouchterlony analysis.

C4: An antiserum to C4 was made by immunization with β_{1E} -anti- β_{1E} precipitin lines cut from immunoelectrophoretic plates. The antiserum gave one unidentified precipitation line as well as the C4 line on Ouchterlony analysis.

C5: An antiserum to C5 was similarly made by injection with precipitin lines between normal euglobulin and an anti-C5 antiserum kindly supplied by Dr. U. Nilsson. It produced a minor precipitin line against C3, as well as a C5 line.

C6: Antiserum to C6 was made by immunizing C6-deficient rabbits with partially purified rabbit C6 (3). The antiserum precipitated well with human serum and was entirely monospecific.

C8: An antiserum to C8 was generously given by Dr. H. J. Müller-Eberhard. It gave precipitin lines with IgG and two unidentified contaminants as well as to C8.

Reactive Hemolysis was demonstrated in 1% agarose plates made up in 0.01 M EDTA in buffered saline and containing 1% sheep cells. (E/EDTA/agarose plates).

Gel filtration on Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden) was carried out in 2.4×60 cm columns using a 0.5 N NaCl buffered with .01 M phosphate pH 7.2, containing 0.01 M azide.

Ultracentrifugation in a sucrose density gradient was carried out in a Spinco Model E ultracentrifuge according to the method of Stanworth (5), using hemoglobin IgG and IgM globulin as internal markers.

Diffusion coefficient in agar was measured by the method of Allison and Humphrey (6) using a rabbit anti-I antiserum. (See Results.)

Chromatography on diethylaminoethyl (DEAE)-cellulose (Whatman DE32) was carried out in 100×2.5 cm columns, in buffers containing 25% or 40% glycerol. In some preparations 0.01 M phosphate buffer, pH 7, was used as starting buffer and gradient elution was performed with the same buffer containing 0.3 N NaCl. In others, 0.01 M phosphate, pH 7.9, was used as starting buffer and gradient elution was performed with 0.25 M NaH_2PO_4 . The elution patterns obtained were essentially similar.

Chromatography on hydroxyapatite was carried out in 40×2.5 cm columns using the buffer systems described by Müller-Eberhard (7).

IgG fractions of various antisera were prepared by column chromatography on DEAE-cellulose (Whatman DE32) of the whole serum equilibrated with 0.015 M phosphate buffer, pH 7.0. The exclusion peak collected and precipitated by 50% saturation with ammonium sulfate.

Complement Compounds.—Partially purified C1 was prepared by the method of Nelson and coworkers (8). Partially purified C2 was prepared by chromatography on carboxymethyl (CM)-cellulose of the supernatant left after precipitation of serum by 20% Na_2SO_4 . Partially purified C4 was prepared by chromatography on DEAE-cellulose of the pseudoglobulin prepared from the 20% Na_2SO_4 precipitate of serum. Partially purified C3 was prepared by chromatography on DEAE-cellulose of the euglobulin from the 20% Na_2SO_4 precipitate. The conditions for the chromatography were substantially similar to those described in (7).

Functionally purified C8 was obtained as a gift from Dr. H. J. Müller-Eberhard.

Intermediate Complexes.—

EAC 142 GP was prepared by incubating EA^1 for 5 min at 37°C with zymosan-adsorbed

¹ Complement nomenclature is used in accordance with the recommendations of the World Health Organization. (1968. *Bull. World Health Organ.* 39:935.)

guinea pig serum (4). These cells were negative in the immune adherence reaction, but were rapidly lysed by EDTA-C.

EAC 1423 (Hu) was prepared by incubating EA for 3 min at 32°C with selected, zymosan-absorbed, human serum (14). The intermediate was positive in immune adherence and aggregated by conglutinin and anti-human C3 sera. Its capacity to be lysed by EDTA-C was very unstable and for lytic tests the cells were used immediately without washing.

EAC 1 ~ 7 was prepared initially by incubating EAC142GP with an appropriate dilution of a human C3 fraction from DEAE-chromatography of euglobulin for 30 min at 37°C. More recently EC567 have been made with activated reactor and functionally purified indicator.

EAC 1 ~ 8. This intermediate was characterized by its ability to be lysed by 0.01 M phenanthroline (9). *EAC 1 ~ 8* were made initially by treating EAC 1 with a DEAE fraction of human pseudoglobulin containing C4-C8. More recently EC5678 cells have been made using functionally purified activated R and DEAE-fractionated indicator preparations (2).

Tests for the Presence of Complement Component Activity.—

C1 was detected by its ability to convert EAC42 to EAC42 \bar{c} ; the latter being lysed by EDTA-C.

C1 inhibitor was tested by the method of Levy and Lepow (10).

C2 was detected by the ability to convert EAC 14 (human) to EAC142 which could then be lysed by EDTA complement.

C4 was tested by the ability to lyse EA in the presence of ammonia-treated guinea pig serum.

C3 was detected by the ability to lyse EAC 142 (guinea pig) in the presence of dilute ammonia-treated guinea pig serum (11).

C6 was detected by the ability to lyse EA in the presence of C6-deficient rabbit serum.

C9 was detected by the ability to lyse EAC 1 ~ 8 intermediates.

Conglutinogen-activating factor (KAF) was measured by its ability to make EAC 1 ~ 3 reactive with conglutinin (17).

RESULTS

The Purification of Indicator Factor.—Euglobulin was prepared from the 20% sodium sulfate precipitate of 500 ml fresh human serum. The elution diagram of this euglobulin preparation from DEAE-cellulose is shown in Fig. 1. It is seen that indicator was eluted maximally at 0.6 millimhos/cm after KAF and before the major protein peak containing C3 (and C6). However, some trailing of indicator was consistently found.

Indicator-containing fractions were pooled and concentrated by ultrafiltration. Antigenically, these fractions contained IgG and C8 as well as indicator.

The indicator fraction from the DEAE column was further fractionated on hydroxyapatite. The I activity was eluted at a conductivity of 11-12 millimhos/cm. Indicator-containing fractions were pooled and concentrated. Antigenically, the pool contained only indicator and some IgG, and was functionally pure.

Physicochemical Characterization of Indicator Factor.—Indicator factor has previously been reported to migrate in agar electrophoresis as a β_1 - β_2 globulin. It was eluted from Sephadex G-200 in the second (7S) protein peak, and quantitation of indicator activity (by the method discussed below) showed that it

occurred maximally on the descending slope of the second protein peak. In the ultracentrifuge, indicator activity sedimented more slowly than IgG (Fig. 2) with a calculated *S* value of 5.7 (mean of three values).

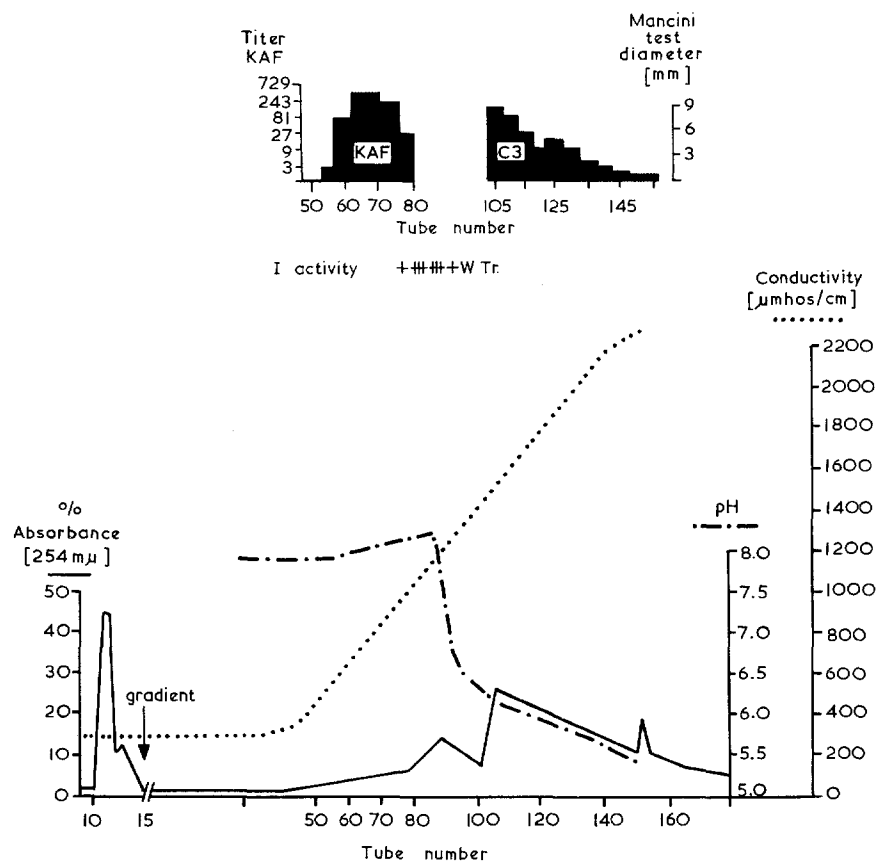


FIG. 1. Fractionation of euglobulin on DEAE-cellulose. 500 mg of human euglobulin prepared from a 20% Na_2SO_4 precipitate of one unit fresh human serum was applied to a 2.5×100 cm DEAE column equilibrated with 0.01 M phosphate buffer, pH 7.9 with 40% glycerol. Gradient elution was performed with 0.25 M NaH_2PO_4 with 40% glycerol. The column was pumped upwards at 30 ml/hr and 12.5 ml fractions were collected.

The diffusion coefficient, as determined by the method of Allison and Humphrey (6) was 4.0×10^{-7} cm^2 per sec. Assuming a value of 0.73 for \bar{v} , the molecular weight was calculated to be about 140,000.

Quantitative Estimation of Indicator Activity.—Indicator could be measured quantitatively by titration, using E and purified preparations of activated reactor (2).

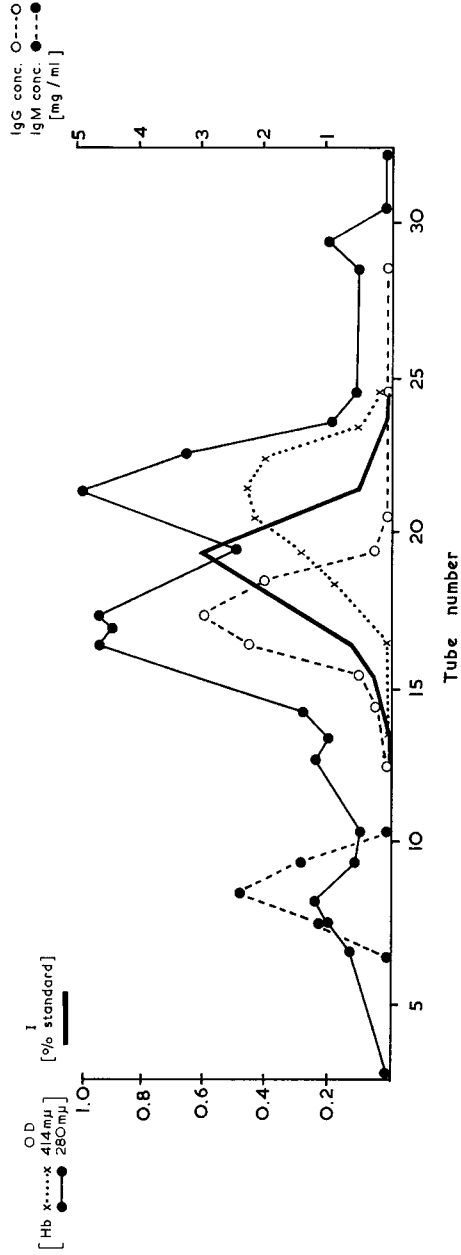


FIG. 2. Sucrose gradient ultracentrifugation of indicator. The sucrose gradient was from 10–40%. Centrifugation was performed for 18 hr at 35,000 rpm in a SW39 Spinco rotor. The bottom of the tube is on the left.

However, this method was time consuming, and much subject to interference by inhibitors. It has been found more convenient to incorporate activated reactor into E/EDTA/agarose plates and to measure indicator by single radial diffusion, simulating the technique of antigen quantitation elaborated by Mancini and coworkers (12).

Holes made in the agarose were filled with sera, or indicator containing preparations, and left for 24 hr at room temperature. Circular zones of partial lysis developed around the holes in which whole sera or indicator preparations were placed (Fig. 3). The diameters of the zones were well defined and easily read to the nearest 0.1 mm, using an eyeglass which incorporated a measuring

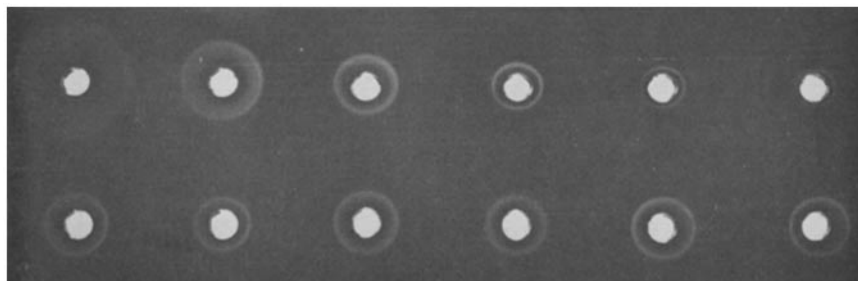


FIG. 3. The single radial diffusion method of quantitation of I. An activated reactor euglobulin was incorporated in to a red cell agarose plate with EDTA. Holes of 2 mm diameter were made in the agar and filled with serial doubling dilutions of a standard human serum (upper row, left to right, 1:2 to 1:64) or dilutions of test sera (lower row, all sera diluted 1:10), or indicator containing preparations. After being left at room temperature for 24 hr, the diameter of the zones of partial lysis around the holes were measured using an eyepiece incorporating a measuring scale.

scale. No lysis occurred in EDTA red cell agarose plates incorporating an activated R preparation which had been destroyed by heating at 56°C or by treatment with ammonia.

The diameters of the zones on any one plate were linearly related to the logarithm of the concentrations of the indicator preparations (Fig. 4). Regardless of its eventual size, the zone of lysis was always partial, being maximal at the outer margin of the circle. Purified indicator preparations showed more complete lysis than whole serum.

This method proved satisfactory for the quantitation of indicator in whole human sera or in fractions. By repeated estimations on 3 separate days in which each of six sera were analyzed 15 times, the standard error of the method was calculated as $\pm 9.1\%$. These estimations were performed with different activated reactor euglobulin preparations incorporated in the plates on each occasion.

Antigenic Characterization of Indicator.—By injecting a rabbit with a partially purified indicator, an antiserum was obtained which gave a number of lines with the injected preparation, the main arcs being β_{1A} and IgG. By selectively cutting out a small precipitation line in the agar in the electrophoretic position of indicator and injecting it in to other rabbits, after the technique described by Goudie et al. (13), an antiserum was obtained which, although not specific, after absorption with a reconstructed freeze-dried Cohn III 2, 3 preparation,

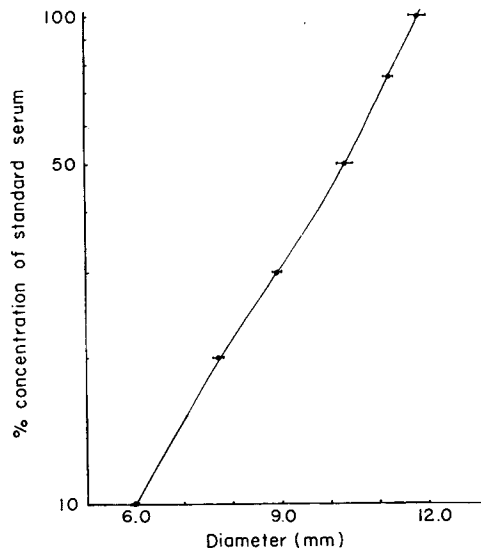


FIG. 4. The relationship between the logarithm of the concentration of a standard serum (ordinate), to the diameter of the zone of reactive hemolysis (abscissa) produced in a red cell agarose EDTA plate incorporating an activated R euglobulin after 24 hr incubation at room temperature. Each point is the mean \pm one SD of six estimations.

showed only a single line in the position of the indicator factor when run against the partially purified indicator. With whole serum, the absorbed antiserum showed one major line which gave a reaction of identity with the line given against the indicator preparation. This major line was antigenically distinct from IgA, IgG, IgM, and IgD, transferrin, β_{1C} - β_{1A} and β_2 glycoprotein.² The commercial polyvalent anti-human serum protein antiserum was also shown to contain antibodies to indicator which gave a reaction of identity with the line produced by the absorbed rabbit antiserum against the partially purified I preparation.

This precipitating antibody was shown to be directed against indicator

² Supplied as a gift from Mr. D. Evans of Behringwerke AG, Marburg-Lahn, West Germany.

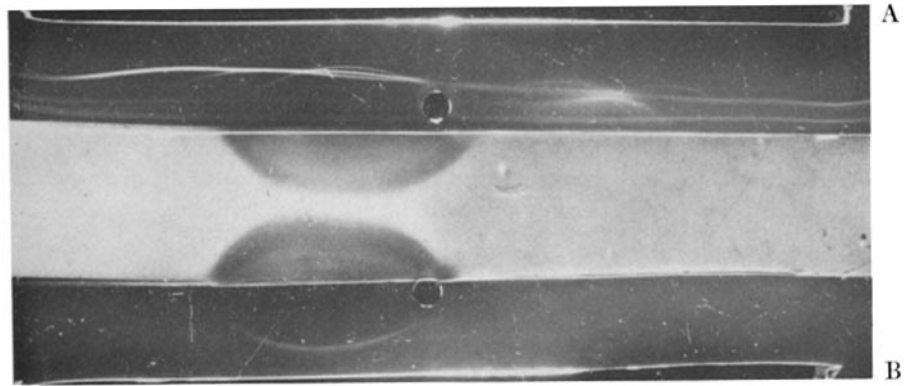


FIG. 5. The relationship of the antigenic I to the hemolytic activity after electrophoresis in agar of a DEAE-I preparation. Anode is to the right. After electrophoresis, the strip of agar was cut out parallel to the direction of separation, as shown, and replaced with a similar sized strip of agarose incorporating sheep red cells, 0.01 M EDTA, and an activated R euglobulin. The upper trough (A) was filled with polyvalent anti-human serum and the lower trough (B) with anti-I. The preparation was photographed after incubation for 18 hr at room temperature.

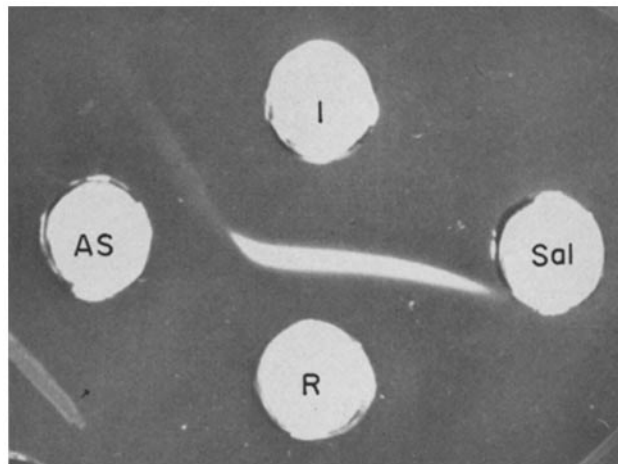


FIG. 6. A section of a red cell agarose plate. The four wells contained indicator (I), activated reactor (R), the IgG fraction of rabbit anti-I antiserum (AS), and saline (S). After 24 hr at room temperature, the plate was photographed to show the hemolysis between I and R. It was washed in 0.15 M NaCl, and then in distilled water to hemolyze the red cells. When the hemoglobin had been washed out of the plate, it was photographed to show the precipitation between I and the antiserum, and the two photographs superimposed to illustrate the action of the anti-I in inhibiting reactive hemolysis.

factor in a number of ways. Firstly, the position of the precipitin lines on immunoelectrophoresis can be shown to correspond to the position of hemolytic indicator activity (Fig. 5). Secondly, IgG prepared from the absorbed antiserum removed hemolytic indicator activity from serum or from indicator-rich fractions, as shown in Fig. 6, where the line of reactive hemolysis stops short at the precipitation line between the indicator and the anti-indicator IgG. IgG similarly obtained from normal rabbit serum or from rabbit anti-human immunoglobulin antisera, did not interrupt the line of reactive hemolysis. The effect of the anti-indicator IgG was specific for human indicator and had no effect on the reaction between activated reactor and the indicator in rabbit

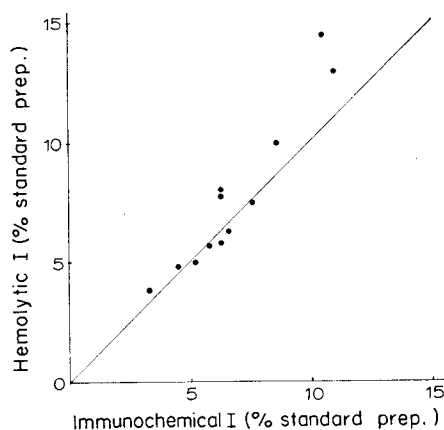


FIG. 7. The comparison of the hemolytic and immunochemical radial diffusion methods of estimating I in 12 different sera. The results are expressed as the percentage of I in the partially purified preparation. In the hemolytic method, dilutions of sera were placed in wells in an E/EDTA/agarose plate incorporating activated R euglobulin, while in the immunochemical method, plain agarose plates incorporating anti-I antiserum were used.

serum or guinea pig serum. Further, by incorporating the antiserum into an agar plate, it was possible to determine the concentration of immunochemical indicator by the single radial diffusion technique, and there was good correlation between the values obtained by this method in 12 sera, with the values for hemolytic activity obtained in the same sera by the "hemolytic radial diffusion" method already described (Fig. 7).

The Interaction of Activated R and I in Plain Agarose plates.—During the analysis of the antigenic characterization of indicator, in plain agar or agarose, it was observed that between activated R and indicator preparations, whether highly purified or in serum, a line invariably developed. This line could be demonstrated by a protein stain, but was never visible as a precipitate in the unstained preparation (Fig. 8). It was not found if the activated R preparations

had been heated for 10 min at 56°C to destroy their hemolytic activity. The line could also be seen by appropriate preparation of a red cell agarose plate and when compared with a photograph of the plate showing the hemolysis, could be seen to occur in the center of the hemolytic zone. Since, in suitable conditions, new hemolytic lines can form through preexisting hemolytic lines, it would seem that the stainable line is not a diffusion barrier, and represents not lattice formation between Activated R and I but the binding of the product of interaction to the gel (see reference 2).

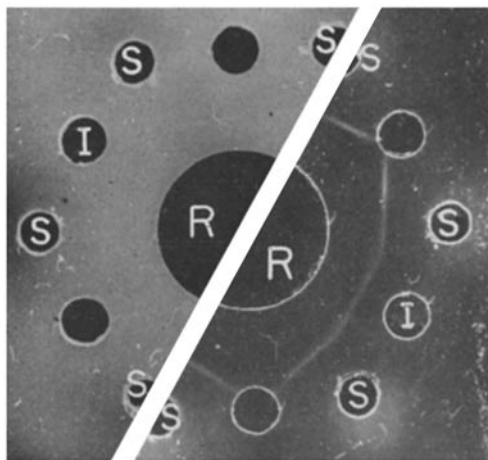


FIG. 8. The "stainable line" between indicator and Activated R. The wells contain functionally purified Activated Reactor (R), purified Indicator (I) and normal human serum (S). The left half picture is of the unstained plate after overnight incubation; the right half picture is of the same plate after washing, drying, and staining.

Characterization of Indicator as a Complement Component.—

(a) *The effect of anti-indicator IgG on complement activity of serum reagents:* Anti-I IgG was found specifically to deplete human EDTA-C of its ability to lyse EAC142 (g.pig) or EAC1423 (Hu) (Table I). Human EDTA serum so treated failed to make EAC 1 ~ 7 cells with EAC142. Similarly, ammonia-treated human serum, after precipitation at optimal proportions with anti-I IgG, would, when tested on EAC14Hu, give rise to EAC142 and EAC1423, but not to the heat stable EAC 1 ~ 7. Finally, activated R preparations which depleted I from EDTA-C at the same time removed its ability to lyse EAC142 and to form EAC 1 ~ 7 from them.

These results suggest that indicator must be either C5, C6, or C7.

(b) *Specific tests for complement component activity:* Tests for presence of specific complement components in purified I preparations were undertaken as

described in the methods section. The I preparation could be shown not to contain C1, C1 inhibitor, C4, C2, C3, C6, or C9 activity. Purified fractions of human C1 and C3 contained negligible amounts of I activity. Purified I gave no precipitation lines with anti-C3, anti-C4, anti-C5, and anti-C6. The sera of mice with a hereditary deficiency of C5 (15) and of rabbits with a deficiency of C6 were both shown to contain I. The purified C8 preparation provided by Dr. H. J. Müller-Eberhard had no I activity, nor did it react with the anti I antiserum, while the anti C8 antiserum failed to react with the functionally purified I preparations. I, therefore, must be C7.

TABLE I

*Effect of DEAE-IgG from Rabbit Anti-Human Indicator Antiserum on Ability of EDTA-C to Lyse EAC1423Hu**

Reagent added to Human EDTA-C	Hemolysis with EAC1423Hu
	%
Saline (control)	61
IgG from anti-I (0.5 mg) antiserum	9
IgG from anti-IgG (2 mg) antiserum	67

* 5×10^8 EA incubated 3 min at 32°C with zymosan-adsorbed human serum diluted 1:10 in Veronal-buffered saline (VBS).

DISCUSSION

The evidence that the indicator factor is a late-acting complement component is strong.

Anti-indicator IgG was able to remove late-acting complement component activity from both EDTA-C and ammonia treated C, and such treated reagents were able to form EAC1423 with EAC142 and EAC14, respectively, but were unable to form EAC 1 ~ 7. The depleted factor must therefore be either C5, C6, or C7. Since indicator is present in C5-deficient mouse serum and C6-deficient rabbit serum, it must be C7. Purified indicator has been shown by a combination of hemolytic and immunochemical tests to contain neither the early complement components, nor C3, C5, C6, C8, or C9. This confirms its identification as C7.

The fractionation and physicochemical data obtained for indicator are compatible with that previously reported for C7 (16), although it is more readily separated from C5 and C6 on DEAE-cellulose than earlier reports have suggested.

A monospecific antiserum to indicator has been made and this has allowed C7 to be recognized after immunoelectrophoresis.

C7 can readily be quantitated as indicator, using a hemolytic radial diffusion

test. This represents a considerable advantage, since conventional hemolytic testing for C7 is difficult and requires purified preparations of a number of complement components.

SUMMARY

This paper describes the characteristics of the indicator factor (I) which takes part in reactive hemolysis and its identification as the seventh component of complement.

I was shown to be a beta globulin with a sediment coefficient of 5.7S and a molecular weight of about 140,000.

Experiments on the depletion of I activity with anti-I antiserum or with activated R euglobulin showed that I was a late acting complement component necessary for the lysis of cells after the EAC142 stage. Complement component analysis of purified I fractions excluded all known components except C7. The physicochemical characteristics of I are compatible with published data on C7. The method of quantitation described represents a convenient method of testing for C7.

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BIBLIOGRAPHY

1. Thompson, R. A., and D. S. Rowe. 1968. Reactive haemolysis—a distinctive form of red cell lysis. *Immunology*. **14**:745.
2. Lachmann, P. J., and R. A. Thompson. 1970. Reactive lysis: the complement mediated lysis of unsensitized cells. II. The characterization of the reactor factor as C5 $\bar{6}$, and the role of C8 and C9. *J. Exp. Med.* **131**:643.
3. Lachmann, P. J. 1969. C6 deficiency in rabbits. XVII Colloquium. Protides of the Biological Fluids. H. Peeters, editor. Pergamon Press. New York. In press.
4. Mayer, M. M. 1961. Complement and complement fixation. In Kabat and Mayer's Experimental Immunochemistry, E. A. Kabat, and M. M. Mayer, editors. Charles C Thomas. Springfield, Ill. 2nd edition. 133.
5. Stanworth, D. R. 1968. Ultracentrifugation of immunoglobulins. In Handbook of Experimental Immunology. D. M. Weir, editor. Blackwell Scientific Publications, Oxford. 44.
6. Allison, A. C., and J. H. Humphrey. 1960. A theoretical and experimental analysis of double diffusion precipitin reactions in gels, and its application to characterisation of antigens. *Immunology*. **3**:95.
7. Müller-Eberhard, H. J. 1969. Methods of isolation of human complement components. In Methods in Immunology and Immunochemistry. M. W. Chase and C. A. Williams, editors. Academic Press, Inc., New York. **3**. In press.
8. Nelson, R. A., Jr., J. Jenson, I. Gigli, and N. Tamura. 1966. Methods for the

- separation, purification and measurement of nine components of hemolytic complement in guinea pig serum. *Immunochemistry*. **3**:111.
9. Hadding, U., and H. J. Müller-Eberhard. 1967. Complement, substitution of the terminal component in immune hemolysis by 1:10 phenanthroline. *Science (Washington)*. **157**:442.
 10. Levy, L. R. and I. H. Lepow. 1959. Assay and properties of serum inhibitor of C1 esterase. *Proc. Soc. Exp. Biol. and Med.* **101**:608.
 11. Klein, P. G., and H. J. Wellensiek. 1965. Multiple nature of the third component of guinea pig complement. I. Separation and characterisation of three factors a, b and c essential for haemolysis. *Immunology*. **8**:590.
 12. Mancini, G., A. O. Carbonara, and J. F. Heremans. 1965. Immunochemical quantitation of antigens by single radial diffusion. *Immunochemistry*. **2**:235.
 13. Goudie, R. B., C. H. M. Horne, and P. C. Wilkinson. 1966. A simple method for producing antibody specific to a single selected diffusible antigen. *Lancet*. **2**:1224.
 14. Lachmann, P. J., and R. Liske. 1966. The preparation and properties of alexinated intermediates that react with conglutinin. II. Equine, rabbit and human complement. *Immunology*. **11**:255.
 15. Nilsson, U. R. and H. J. Müller-Eberhard. 1967. Deficiency of the fifth component of complement in mice with an inherited complement defect. *J. Exp. Med.* **125**:1.
 16. Müller-Eberhard, H. J. 1968. Chemistry and reaction mechanisms of complement. *Advan. Immunol.* **8**:2.
 17. Lachmann, P. J., and H. J. Müller-Eberhard. 1968. The demonstration in human serum of 'conglutinin activating factor' and its effect on the third component of complement. *J. Immunol.* **100**:691.