CHROMATOGRAPHIC RESOLUTION OF THE FIRST COMPONENT OF HUMAN COMPLEMENT INTO THREE ACTIVITIES*

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Serum complement has traditionally been defined as a four component system, C'1, C'2, C'3, and C'4. Although several investigators had suggested the existence of additional components (for review of earlier literature, see reference 1), only recently has unequivocal evidence been available supporting the further complexity of complement. The third component, C'3, was shown first to consist of at least two activities (1–4) and then three (5, 6), designated C'3a, C'3b, and C'3c. A highly purified protein, β_{1c}-globulin (7), would appear to be identical with C'3a (5).

Müller-Eberhard and Kunkel (8, 9) and Taranta, Weiss, and Franklin (10) have described a new serum protein, distinct from the classical components of complement and designated the 11S component on the basis of its sedimentation constant. In earlier work on the sequence of action of the components of complement (11, 12), the first component, C'1, was the first factor recognized to interact with sensitized erythrocytes. However, evidence has been presented that the 11S component participates in immune hemolysis at a stage even earlier than C'1 (8, 9), an observation which would appear to require revision of previous concepts of sequence.

It is the purpose of this paper to report the resolution of C'1 into three activities by chromatography on diethylaminoethyl (DEAE) cellulose of a euglobulin fraction of normal human serum. It will be shown that all three factors are required for reconstitution of C'1 activity in several hemolytic systems and for generation of C'1 esterase in the absence of antigen-antibody complexes (13). Data will be presented supporting the identity of one of these

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factors with the 11S component (8–10) and another with C'1 proesterase, the proenzyme associated with C'1 and capable of activation to C'1 esterase (14, 15). The third factor, previously unrecognized, represents a new component of complement. The three factors, eluted from DEAE cellulose in the order (*a*) 11S component, (*b*) new component, and (*c*) C'1 proesterase, are tentatively designated C'1q, C'1r, and C'1s, respectively. In view of these findings, the 11S component may be considered as one part of the activity originally ascribed to C'1, an interpretation which requires revision of detail but not of substance of previous investigations on the earliest steps of complement action (11, 12).

Materials and Methods

Human Serum.—Human blood collected without anticoagulant was allowed to clot at room temperature for several hours and was stored at 1°C overnight for maximal clot retraction. The serum was separated by centrifugation at 1°C. Pools of serum from 10 to 100 donors were frozen in aliquots at −70°C.

Guinea Pig Serum.—Guinea pig blood was obtained by cardiac puncture and the serum separated and stored as described for human serum.

Salts of Ethylenediaminetetraacetic Acid (EDTA).—Reagent grade Na_2H_2EDTA was titrated to pH 7.4 at a stock concentration of 0.15 M, giving a solution largely in the form Na_3HEDTA. $Na_2MgEDTA$ (Geigy Chemical Corp., Ardsley, New York) was titrated to pH 7.4 at a stock concentration of 0.15 M.

Buffer Diluents for Complement Assays.—Triethanolamine-buffered saline (TBS) at pH 7.4, ionic strength 0.15, containing 1.5×10^{-4} M Ca^{++} and 5×10^{-4} M Mg^{++} (16), was employed as the diluent in all assays involving a hemolytic system other than the Donath-Landsteiner reaction. In the latter case, barbital buffer of comparable constitution was used (12). TBS-gelatin buffer was prepared by making a final concentration of 0.05 per cent gelatin (Knox special intravenous, Knox Gelatine Protein Products, Inc., Camden, New Jersey) in TBS buffer. TBS-gelatin-Na_3HEDTA buffer contained 8×10^{-3} M Na_3HEDTA. TBS-gelatin-$Na_2MgEDTA$ buffer contained 16×10^{-3} M $Na_2MgEDTA$.

Soy Bean Trypsin Inhibitor.—The five times–recrystallized inhibitor (Nutritional Biochemicals Corporation, Cleveland) was suspended in TBS buffer at a concentration of 15 mg per ml or weighed and transferred to reaction vessels for suspension in the reactants.

Sensitized Sheep Erythrocytes (EA).—Sterile sheep erythrocytes in Alsever's solution were washed, standardized spectrophotometrically to a concentration of 10^9 per ml, and sensitized with an equal volume of an appropriate dilution of rabbit hemolysin. Two types of hemolysin were used: glycerinated anti–sheep erythrocyte rabbit serum (Cappel Laboratories, West Chester, Pennsylvania); and anti–Forssman serum prepared by immunization of rabbits with boiled sheep erythrocyte stromata (in part, kindly supplied by Dr. M. M. Mayer). When using commercial antiserum, sensitization was performed with 4 units of hemolysin, as determined by the method described in Kabat and Mayer (17). In the case of anti–Forssman rabbit serum, optimal sensitization was achieved by the method described by Mayer (12). Since glycerinated hemolysin contains variable amounts of rabbit C'1 not inactivated by heating at 56°C for 30 minutes (18), sensitization was performed in the presence of 8×10^{-3} M Na_3HEDTA, followed by three washes with TBS-gelatin buffer. C'1 in the non-glycerinated anti–Forssman rabbit serum was inactivated by heating at 56°C for 30 minutes and the presence of Na_3HEDTA was, therefore, not necessary when the latter hemolysin was used for sensitization. For reasons to be discussed below, suspensions of EA prepared with heated anti-Forssman rabbit serum were washed twice with equal volumes of TBS-gelatin buffer.

Human Complement Reagents.—The four well recognized components of complement are indicated by the symbols C'1, C'2, C'3, and C'4. Serum fractions or reagents which are deficient in one of these components are designated R1, R2, R3, and R4, respectively, and were prepared essentially as described by Mayer (12) with modifications summarized by Wedgwood (19). The 11S component and the serum reagent deficient in 11S component, designated R11S, were prepared according to the procedures of Müller-Eberhard and Kunkel (8), modified as follows: (*a*) in the preparation of aggregated γ-globulin, one-tenth volume of pH 8 sodium phosphate buffer, ionic strength 0.15, was added to 0.15 M NaCl; (*b*) the aggregated γ-globulin was used at a concentration of 20 O.D. units/ml (280 mμ); one-fifth volume relative to serum was added in preparation of 11S component, whereas an equal volume was added to make R11S; (*c*) Spinco ultracentrifugation was in a No. 40 rotor at 40,000 RPM for 60 minutes in the preparation of 11S component, and 120 minutes in the preparation of R11S.

Complexes between Complement and Sensitized Sheep Erythrocytes.—

EA-huC'$_{1,4,2}$. This intermediate complex between human complement and EA having the activities of C'1, C'4, and C'2 was prepared with human R3 according to Leon (20). The activity of this complex can be quantified by measuring the extent of hemolysis by human serum in the presence of 8×10^{-3} M Na_3HEDTA. Na_3HEDTA chelates Ca^{++} and Mg^{++}, blocking the action of C'1, C'4, and C'2 but permitting reaction of C'3 (21).

EA-huC'$_{1,4}$. This intermediate complex between human complement and EA having the activities of C'1 and C'4 was prepared by inactivating the C'2 activity of washed EA-huC'$_{1,4,2}$ at 37°C for 40 minutes (22). The activity of this complex can be quantified by measuring the extent of hemolysis by human serum containing $Na_2MgEDTA$. $Na_2MgEDTA$ chelates Ca^{++} but not Mg^{++} and therefore permits C'2 and C'3 to function while blocking the activity of C'1 (21). For consistent results, a concentration of 16×10^{-3} M $Na_2MgEDTA$ was necessary.

EA-huC'$_4$. This intermediate complex between human complement and EA having the activity of C'4 was prepared by eluting C'1 from EA-huC'$_{1,4}$ (23, 24). The elution was performed twice with 5×10^{-3} M Na_3HEDTA in TBS-gelatin buffer at 37°C for 15 minutes, using 10 ml of Na_3HEDTA with the pellet from 100 ml of the complex EA-huC'$_{1,4}$. This complex, which will *not* hemolyze in the presence of human serum containing 16×10^{-3} M $Na_2MgEDTA$, may be employed for detection of C'1. Incubation of a solution containing C'1 with EA-huC'$_4$ will result in formation of the complex EA-huC'$_{1,4}$ which can be measured as indicated above. In this situation, the extent of hemolysis of EA-huC'$_{1,4}$ by human serum containing 16×10^{-3} M $Na_2MgEDTA$ is a measure of the C'1 activity of the original test solution.

EA-huC'$_1$. This intermediate complex between human complement and EA having the activity of C'1 was prepared by direct reaction of EA with a source of C'1 deficient in C'4 (23, 25–27). The activity of this complex can be quantified by measuring the extent of hemolysis either by R1 or by human serum containing 16×10^{-3} M $Na_2MgEDTA$. R1 contains C'2, C'3, and C'4 but is deficient in C'1 while $Na_2MgEDTA$ chelates Ca^{++}, blocking further action of C'1, but permitting reaction of C'2, C'3, and C'4.

Measurement of Components of Complement.—The components *C'1*, *C'2*, *C'3*, and *C'4* were estimated in indicated instances by serial dilution of samples and measurement of the extent of hemolysis when added to EA in the presence of R1, R2, R3, and R4, respectively (12). The actual methods used and the definition of units have been given in detail elsewhere (13, 28). *C'1* was also measured by its ability to form the complex EA-huC'$_{1,4}$ from EA-huC'$_4$, and to form directly the complex EA-huC'$_1$. *C'4* in partially purified preparations was estimated by the serial dilution method, using R4 fortified with a source of C'3a. *C'3a* was measured by the amount of hemolysis obtained when the complex EA-huC'$_{1,4,2}$ was incubated with the test sample in the presence of a 1/4000 dilution of normal guinea pig serum con-

taining 8×10^{-3} M Na_3HEDTA (29). *11S Component* was assayed by its ability to reconstitute the hemolytic activity of R11S. One unit of 11S component activity was defined as the least volume of test fraction capable of causing 50 per cent hemolysis of 1.0 ml of EA (2.5×10^8) in the presence of 0.08 to 0.10 ml of R11S during incubation at 37°C for 30 minutes. The preparation of 11S component used in the experiments to be described contained 60 units/ml.

All assays involving intermediate complexes were performed in baths controlled to ±0.1°C, using a wrist-action shaker for mixing. The final lytic reaction in these cases was performed at 32°C for 60 minutes and the extent of hemolysis measured by reading the optical density at 541 mμ of the supernatant solution.

Donath-Landsteiner Reaction.—The Donath-Landsteiner reaction, an immune hemolytic system entirely of human origin, has provided a convenient model for study of the mechanism of action of human complement. The antibody is contained in serum from patients with paroxysmal cold hemoglobinuria (PCH). The reaction is biphasic in nature, requiring initially interaction of erythrocytes, antibody, and C'1 at 1°C. Hemolysis is completed after subsequent interaction with the remaining components of complement.

The reaction was performed as described previously (30, 31). The Donath-Landsteiner antibodies used were contained in serum heated at 56°C for 30 minutes, or in fractions prepared chromatographically (30). Erythrocytes from a patient with paroxysmal nocturnal hemoglobinuria (PNH) were used throughout because of their greater sensitivity to hemolytic antibodies. In the cold phase of the reaction, 0.05 ml of a 25 per cent suspension of PNH erythrocytes, 0.05 to 0.10 ml of antibody, 0.10 ml volumes of complement-containing fractions, and barbital buffer were incubated together in a volume of 0.35 to 0.50 ml at 1°C for 30 minutes. The cells were washed twice at 1°C with barbital buffer, 0.30 ml of R1 was added, and the mixture was incubated at 37°C for 30 minutes. Hemolysis in the supernatant serum was measured at 541 mμ.

Enzymatic Assay of C'1 Esterase.—The esterolytic activity of C'1-esterase was measured using *N*-acetyl-L-tyrosine ethyl ester as substrate. One unit of C'1 esterase is defined as that amount which liberates 0.5 micromole of H^+ in 15 minutes at 37°C as detected by microformol titration with 0.05 N NaOH (32, 33).

Ion Exchange Chromatography.—Diethylaminoethyl (DEAE) cellulose, (high capacity, type 20, exchange capacity 1.05 meq/gm, Carl Schleicher and Schüll Co., Keene, New Hampshire) was suspended in 30 times its weight of water, stirred vigorously, and allowed to settle for several hours. The supernatant fluid, containing fine particles, was decanted, and the washing was repeated until the supernatant fluid was completely clear after 30 minutes of settling. Washing was continued on a coarse sintered-glass funnel with a solution containing 0.125 M NaOH and 0.125 M NaCl, followed by water until washings were alkali-free. The washed adsorbent was suspended in starting buffer for packing into chromatographic tubes. The starting buffer in all cases was sodium phosphate, pH 7.4, ionic strength 0.15, containing 1×10^{-3} M Na_3HEDTA.[1]

Columns were prepared from chromatography tubes (internal diameter 2.5 cm) by forcing a magnetically stirred slurry of DEAE cellulose (10 to 20 gm/liter) into the tube under 5 pounds per square inch air pressure until the packed height of adsorbent was 25 cm. The column was transferred to a cold room (5°C), washed with several liters of cold starting buffer, and stored in starting buffer until used.

After chromatography was completed, the DEAE cellulose was regenerated in the columns by flushing with 3 to 4 column volumes of a solution containing 0.125 M NaOH and 0.125 M

[1] 50 ml of 0.2 M NaH_2PO_4 was mixed with 233.5 ml of 0.2 M Na_2HPO_4 and 6.67 ml of 0.15 M Na_3HEDTA at pH 7.4. This solution was then diluted to 1000 ml with distilled water.

NaCl, followed by 2 column volumes of 1 per cent (v/v) HCl, and starting buffer until the pH of the effluent was the same as the starting buffer. The use of dilute HCl was found necessary for only every other chromatographic run with each column. Because of decreasing flow rates with successive use, it was found expedient to empty the columns after every 5 or 6 experiments and repack the tubes with fresh adsorbent.

EXPERIMENTAL

I. Preparation and Properties of a Euglobulin Fraction of Normal Human Serum Containing C'1 Activity

A. Preparation of Euglobulin.—The preparation and properties of a partially purified fraction of human C'1 have been described previously (13, 14, 32). A simpler preparative procedure, yielding greater recovery of C'1 but less initial purification, was devised for these investigations. The method is a modification of the procedure for preparing R2, the serum euglobulin fraction deficient in C'2, by dilution and acidification (12).

One volume of human serum at 1°C was added with constant stirring to 8 volumes of acetate buffer, pH 5.5, ionic strength 0.02, at 1°C. The mixture was allowed to stand undisturbed at 1°C overnight and the supernatant fluid was then withdrawn by siphon. The residual precipitate was centrifuged at 4000 RPM for 30 minutes at 1°C and washed at 1°C with a volume of acetate buffer, pH 5.5, ionic strength 0.02, equal to the original volume of serum. The washed precipitate was suspended in 0.5 M NaCl containing 10^{-3} M Na_3HEDTA to one-tenth the original volume of serum and centrifuged cold at 30,000 RPM for 60 minutes in a No. 30 rotor in the Spinco preparative ultracentrifuge. The supernatant fluid was poured through a filter of glass-wool to exclude particles of lipid and the filtrate was dialyzed at 1°C *vs.* 50 volumes of pH 7.4 phosphate buffer, ionic strength 0.15, containing 10^{-3} M Na_3-HEDTA. After 12 to 18 hours, the dialyzed fraction was centrifuged at 4000 RPM for 30 minutes at 1°C. The resulting opalescent solution, concentrated 10-fold with respect to serum, represented the crude euglobulin fraction of C'1 employed as starting material for column chromatography.

B. Properties of Euglobulin Fraction as a Source of C'1 in Hemolytic Systems.—*Yield and purification of C'1:* Employing a serial dilution assay with R1 and sensitized sheep erythrocytes (EA), 80 per cent of the C'1 activity present in the original serum was recovered in the euglobulin fraction. The purification of C'1 was 20-fold with respect to serum on the basis of nitrogen determinations, and 16-fold on the basis of absorption at 280 mμ. Again employing serial dilution assays with R2, R3, or R4, recoveries of other components were: C'2, <1 per cent; C'3, 27 per cent; and C'4, 2 per cent. C'1 activity, measured as described above, was stable at 1°C, − 20°C, and −60°C for at least 2 weeks. This prolonged stability of C'1 at pH 7.4, ionic strength 0.15, was dependent upon the presence of Na_3HEDTA, previously shown to inhibit the activation of C'1 to C'1 esterase (13).

C'1 activity as measured by formation of the complex EA-huC'$_{1,4}$:

The complex EA-huC'$_4$ may be prepared as shown schematically in the following reactions:

$$EA + R3 \xrightarrow{Ca^{++}, Mg^{++}} EA\text{-}huC'_{1,4,2}$$

$$EA\text{-}huC'_{1,4,2} \xrightarrow{37°C} EA\text{-}huC'_{1,4}$$

$$EA\text{-}huC'_{1,4} \xrightarrow{Na_2HEDTA} EA\text{-}huC'_4 + C'1 \text{ eluate}$$

The resulting complex EA-huC'$_4$, washed free of C'1 eluate, is not hemolyzed by serum diluted in $Na_2MgEDTA$ buffer but is hemolysed by R4. If these criteria are met, the complex EA-huC'$_4$ may be used for detection and measurement of C'1 in unknown preparations. For example:

$$EA\text{-}huC'_4 + \text{euglobulin} \xrightarrow{Ca^{++}} EA\text{-}huC'_{1,4}$$

$$EA\text{-}huC'_{1,4} + \text{human serum} \xrightarrow{Na_2MgEDTA} \text{hemolysis}$$

Pellets of the complex EA-huC'$_4$ containing 5×10^8 cells were suspended in 1 ml of various dilutions of recalcified euglobulin. The mixtures were incubated for 15 minutes at 37°C, washed with 5 ml of cold TBS-gelatin buffer, and suspended in 4 ml of human serum diluted 1/100 in $Na_2MgEDTA$ buffer. The extent of hemolysis was read at 541 mμ after incubation at 32°C for 60 minutes.

The euglobulin fraction of C'1 was highly active in forming the complex EA-huC'$_{1,4}$ from the complex Ea-huC'$_4$. As shown in Fig. 1, the extent of formation of the complex EA-huC'$_{1,4}$ was a sigmoidal function of the relative concentration of C'1 supplied by the euglobulin. Less than 1 μg of nitrogen supplied sufficient C'1 to yield EA-huC'$_{1,4}$ complexes with significant hemolytic activity.

C'1 Activity as measured by formation of the complex EA-huC'$_1$: The complex EA-huC'$_1$ may be prepared from sensitized sheep erythrocytes and a source of C'1:

$$EA + C'1 \xrightarrow{Ca^{++}} EA\text{-}huC'_1$$

The resulting complex may be measured by hemolysis with R1 or human serum diluted in $Na_2MgEDTA$ buffer.

Pellets of EA containing 5×10^8 cells were suspended in 1 ml of various dilutions of recalcified euglobulin. The mixtures were incubated for 10 minutes at 37°C. (In separate experiments it was found that EA-huC'$_1$ formation was maximal at 37°C within several minutes and was not affected by further incubation for periods as long as 30 minutes.) After incubation the mixtures were centrifuged, washed with 5 ml of TBS-gelatin buffer at 37°C,[2] and suspended

[2] It is critically important that the complex EA-huC'$_1$ be washed at an elevated temperature such as 37°C rather than in the cold. The authors are grateful to Dr. M. A. Leon for this highly pertinent observation (27).

in 4 ml of human serum diluted in $Na_2MgEDTA$ buffer. The extent of hemolysis was read at 541 mμ after incubation at 32°C for 60 minutes.

The euglobulin fraction was highly active in forming the complex EA-huC'$_1$. As shown in Fig. 1, the extent of formation of the complex EA-huC'$_1$ was a sigmoidal function of the relative concentration of C'1 supplied by the euglobulin. The activity of the euglobulin in forming the complex EA-huC'$_1$ was approximately the same as that presented earlier for formation of EA-huC'$_{1,4}$ from EA-huC'$_4$.

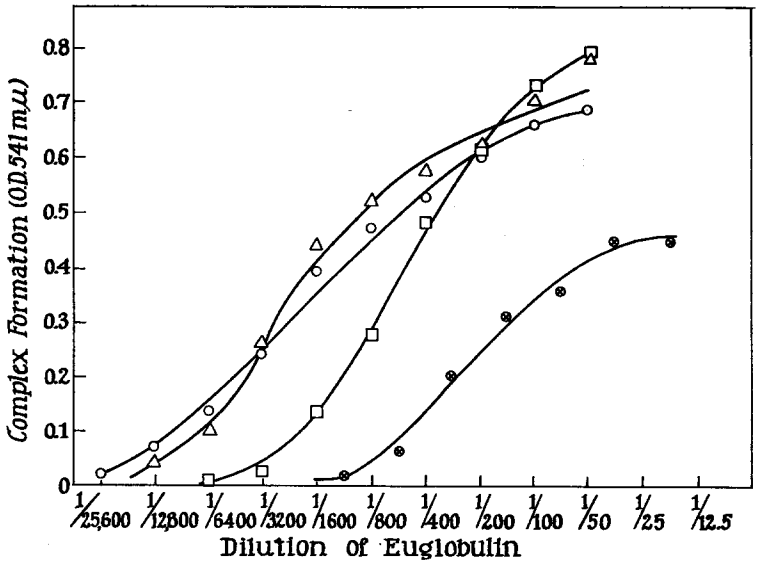

FIG. 1. C'1 activity of euglobulin fraction in forming intermediate complexes: ○, EA-huC'$_{1,4}$ from EA-huC'$_4$; △, EA-huC'$_1$, using normal euglobulin fraction; □, EA-huC'$_1$, using euglobulin fraction prepared from R4; ⊗, cold phase of the Donath-Landsteiner reaction.

This experiment was compromised by the presence of small amounts of C'4 in the euglobulin fraction and the possibility that the complex formed, in fact, represented EA-huC'$_{1,4}$. In order to demonstrate unequivocally that the euglobulin fraction was capable of forming EA-huC'$_1$, the above experiment was repeated using a euglobulin prepared from R4. As shown in Fig. 1, this preparation, completely devoid of detectable C'4, was active in forming the complex EA-huC'$_1$. The significance of quantitative difference in activities of euglobulin fractions, tested at different times with different lots of EA, has not been investigated.

C'1 activity as measured in the cold phase of the Donath-Landsteiner reaction: The Donath-Landsteiner reaction requires the presence of components of

complement in the initial cold phase of the reaction as well as in the second or warm phase. Previous studies indicated that C'1 reacts with cells and antibody in the initial phase (31). The euglobulin fraction was therefore tested for its ability to complete the cold phase.

Serial dilutions of euglobulin (0.10 ml) were assayed as usual in the Donath-Landsteiner reaction, employing heated serum as a source of PCH antibody. The results, plotted in Fig. 1, demonstrated that the euglobulin fraction was a satisfactory source of C'1, as measured by this parameter of C'1 activity.

C. Properties of Euglobulin Fraction as a Source of C'1 Esterase.—When the euglobulin fraction was recalcified to a final concentration of 10^{-3} M $CaCl_2$ and incubated at 37°C, C'1 esterase activity was generated and hemolytic C'1 activity disappeared (double dilution assay with R1) at parallel and rapid rates. For example, a euglobulin fraction containing 20,000 units of C'1/ml was recalcified and diluted 1/3 in TBS buffer. C'1 activity completely disappeared and maximal generation of C'1 esterase (180 units/ml of undiluted euglobulin) occurred during incubation at 37°C for 10 minutes and was unaffected by further incubation. Both reactions were approximately half maximal at 5 minutes. Thus, with respect to these properties, the euglobulin fraction of C'1 behaved in a manner qualitatively similar to that described previously (13) for a somewhat more purified preparation.

D. Summary of Properties of Euglobulin Fraction as a Source of C'1.—The preparation of euglobulin met the following criteria for C'1 activity: (*a*) reconstitution of the hemolytic activity of R1; (*b*) formation of the complex EA-huC'$_{1,4}$ from EA-huC$_4$; (*c*) direct formation of the complex EA-huC'$_1$; (*d*) completion of the cold phase of the Donath-Landsteiner reaction; and (*e*) generation of C'1 esterase during incubation at physiologic conditions of pH and ionic strength. On the basis of these criteria, the euglobulin fraction represented a satisfactory source of C'1 for further fractionation by column chromatography.

II. Chromatographic Resolution of the Euglobulin Fraction into Three Activities of C'1

A. Column Chromatography on DEAE Cellulose.—Chromatography was performed at 5°C. Twenty ml of human serum euglobulin fraction, concentrated 10-fold with respect to serum, was allowed to drain into a packed, equilibrated, DEAE cellulose column. Residual protein was flushed into the column with several 2 ml aliquots of cold starting buffer (pH 7.4 phosphate, ionic strength 0.15, containing 1×10^{-3} M Na_3HEDTA) and cold starting buffer was allowed to flow through the column. The effluent was collected in 10 ml aliquots on a volumetric fraction collector. A linearly increasing gradient of salt concentration at constant pH was run into the column after 100 to 150 ml had been collected. The gradient was produced by allowing 1000 ml of limit buffer (0.5 M NaCl in starting buffer, 1×10^{-3} M Na_3HEDTA final concentration) in a liter Erlenmeyer flask to flow through a capillary tube into an identical mixing flask containing starting buffer in hydrostatic equilibrium with the contents of the

first flask, and thence into the column. The mixing flask was stirred magnetically. Usually 115 to 125 aliquots were collected at a flow rate of 30 ml/hour under gravity flow. The protein content of each tube was estimated by measuring the absorption at 280 mμ in a Zeiss spectrophotometer, care being taken to expose each fraction to room temperature as briefly as possible. Graphic plots were made of fraction number *vs.* protein eluted, expressed as optical density at 280 mμ.

Over twenty chromatographic separations were performed under these conditions, using two different lots of DEAE cellulose, each of the same type

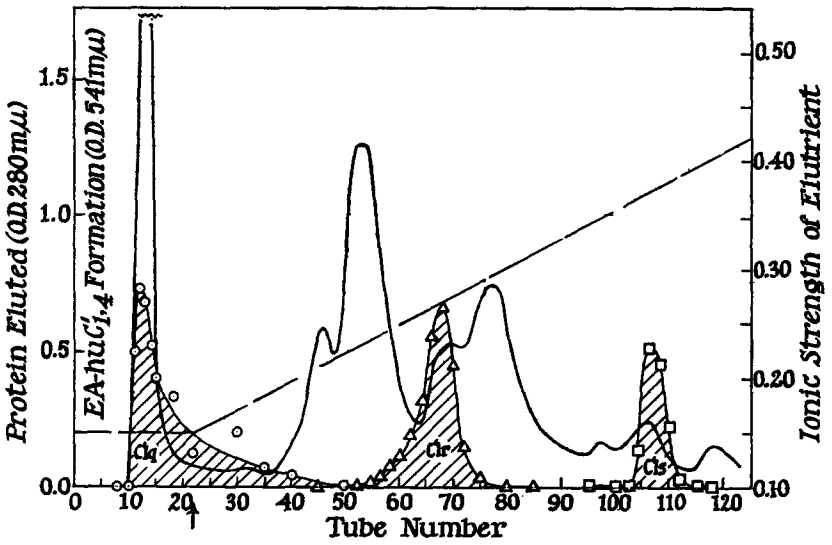

FIG. 2. Chromatographic resolution of C'1 activity of euglobulin fraction into three activities: formation of EA-huC'$_{1,4}$ from EA-huC'$_4$; ——, protein eluted; — —, ionic strength of elutrient; cross-hatched areas, C'1 activities. The arrow indicates the start of the salt gradient.

and with the same exchange capacity. The euglobulin preparations were made from six different pools of normal human serum. Although minor differences were observed in the protein elution patterns in these experiments (for example, compare Figs. 2 and 3), the separation and positions of the activities to be described were remarkably similar. Protein recovery from the column was approximately 80 per cent.

B. Properties of the Chromatographic Fractions in Hemolytic Systems.—Preliminary experiments with R1: Individual column fractions, recalcified with 5×10^{-4}M excess of $CaCl_2$, were tested initially for C'1 activity by serial dilution assay with R1. Significant amounts of C'1 were not detected, a finding not attributable, by direct measurement, to generation of C'1 esterase during

chromatography. Two additional possibilities were entertained: either C'1 activity had been inactivated during fractionation or C'1 had been resolved into more than one chromatographic species. In order to test the latter possibility, various combinations of chromatographic fractions were tested for their hemolytic activity with R1. Referring to Fig. 3 as a typical chromatogram, it was found that addition to R1 of small amounts of a fraction in the region of tube 60 (0.05 ml of fraction + 0.15 ml of R1) permitted the measurement of some C'1 activity in the region of tube 100. However, the reagent itself (frac-

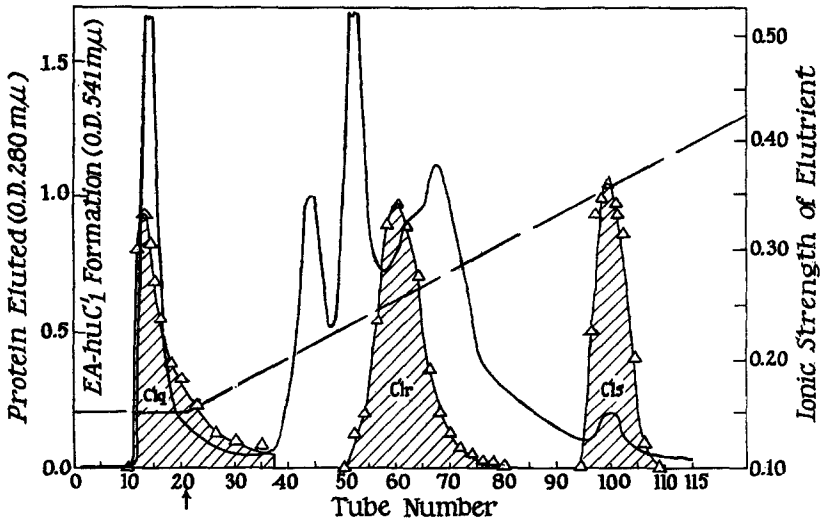

FIG. 3. Chromatographic resolution of C'1 activity of euglobulin fraction into three activities: formation of EA-huC'1; ——, protein eluted; — —, ionic strength of elutrient cross-hatched areas, C'1 activities. The arrow indicates the start of the salt gradient.

tion 60 + R1) was usually 5 to 20 per cent hemolytic and complete hemolysis of EA could not be achieved even with high concentrations of fraction 100. Further preliminary experiments indicated the possible participation of an additional factor in the region of tube 12 for reconstitution of the hemolytic activity of R1. However, control levels of hemolysis with R1 to which fractions from two suspected areas of activity had been added (*e.g.* fractions 12 + 68) were too great to permit rigorous conclusions to be drawn. Nevertheless, these studies with R1 suggested that failure to measure C'1 in individual column fractions was due to resolution of C'1 into more than one activity. The problem was therefore investigated further and with greater precision by the use of intermediate complexes of the immune hemolytic system.

C'1 activity as measured by formation of the complex EA-huC'$_{1,4}$: Individual column fractions, recalcified with 5×10^{-4}M excess of $CaCl_2$, were tested for

C'1 by their ability to form the complex EA-huC'$_{1,4}$ from EA-huC'$_4$. The fractions were assayed using the conditions described above for the euglobulin fraction, except that the incubation time for complex formation was reduced to 10 minutes and was followed by washing with TBS-gelatin buffer at 37°C. Formation of the complex EA-huC'$_{1,4}$ was not observed with any individual fraction or combination of two fractions tested at dilutions of 1/100. However, combination of fractions from three discrete areas of the chromatogram resulted in formation of large amounts of the complex EA-huC'$_{1,4}$. Referring to Fig. 2, these areas were in the region of tubes 12, 68, and 106.

The activity peaks shown were obtained in the following fashion: pellets of the complex EA-huC'$_4$ containing 5×10^8 cells were suspended in 0.5 ml each of 1/200 dilutions of fractions 12 and 68 and 0.5 ml of 1/400 dilutions of fractions from the remainder of the chromatogram. Formation of the complex EA-huC'$_{1,4}$ was first detected with fraction 104, was maximal with fraction 106, and was no longer detectable with fraction 115. The quantitative results of this experiment are plotted in Fig. 2 as the activity peak on the far right, corresponding to a protein peak eluted at ionic strength 0.37.

In a similar fashion, the activity peak shown in the center of Fig. 2, was obtained by repeating this type of experiment using 1/200 dilutions of fractions 12 and 106 with 1/400 dilutions of fractions from the remainder of the chromatogram. Detectable activity was confined to the region between tubes 55 and 80 with maximal activity at tube 68. This corresponded to a chromatographic species eluted at ionic strength 0.27.

Finally, the activity peak shown on the left of Fig. 2 was obtained using 1/200 dilutions of fractions 68 and 106 and 1/400 dilutions of fractions from the remainder of the chromatogram. Activity was maximal with fraction 12, corresponding to the protein peak of the pregradient fraction not adsorbed to DEAE cellulose at ionic strength 0.15. Although most of the activity was confined to the pregradient peak, small amounts were detectable in fractions eluted at ionic strength 0.15 to 0.20.

Combination of fractions 68 and 106 at 1/200 dilutions did not result in most instances in significant formation of the complex EA-huC'$_{1,4}$; that is, levels of hemolysis (O.D., 541 mμ) were in the range of 0.020 to 0.030, to be compared with 0.010 to 0.020 for the EA-huC'$_4$ complex itself. However, it was an inconstant finding that such combinations sometimes yielded greater hemolysis, even when using the same fractions and same preparation of EA-huC'$_4$ complex. Employing 1/100 dilutions of fractions 68 and 106, levels of hemolysis as high as 0.145 have been observed, to be compared with 1.10 for combinations of fractions 12, 68, and 106 at 1/100 dilutions. An adequate explanation of the inconstancy of this observation is not available at the present time.

These experiments demonstrated that the C'1 activity present in the euglobulin fraction necessary for formation of the complex EA-huC'$_{1,4}$ from EA-huC'$_4$ could be chromatographically resolved into at least three separate activities. For purposes of description, these activities will be designated C'1q, C'1r, and C'1s in the order of their elution from DEAE cellulose.

C'1 activity as measured by the complex EA-huC'$_1$: Recognition of the multiple nature of C'1 led to uncertainties concerning the actual state of the complex EA-huC'$_4$. It will be recalled that this complex is made by decay of the complex EA-huC'$_{1,4,2}$, followed by elution of C'1 with Na_3HEDTA. Although C'4, but no other activities, could be measured on the resulting complex by conventional means, the possibility was considered that unrecognized components might be present. It was therefore of interest to attempt to form the complex EA-huC'$_1$ directly from EA and various fractions from the DEAE cellulose columns.

The results of such experiments were completely analogous to those just described for formation of the complex EA-huC'$_{1,4}$. Using the technique described above for formation of the complex EA-huC'$_1$ by reaction of the euglobulin preparation with EA, fractions from three discrete areas of the chromatogram were again required. The activity peaks for C'1q, C'1r, and C'1s shown in Fig. 3 were obtained in the manner already described, except that the activity sought was tested at a dilution of 1/200 using 1/100 dilutions of the remaining two components.

The lack of participation of C'4 in these reactions involving C'1q, C'1r, and C'1s was demonstrated by chromatographing a euglobulin fraction prepared from R4. C'1q, C'1r, and C'1s activity peaks were present in the usual positions and were active in forming the complex EA-huC'1, despite the complete absence of C'4.

Parallel assays in which the same chromatographic fractions were used either for formation of the complex EA-huC'$_{1,4}$ or the complex EA-huC'$_1$ demonstrated an excellent correlation between these two methods of measurement of C'1q, C'1r, and C'1s. In each case, the three activities were present in the same areas of the chromatogram and maximal activities were found in the same fractions.

These results suggested that elution of the complex EA-huC'$_{1,4}$ with Na_3HEDTA at 37°C resulted in removal from the cell of C'1q, C'1r, and C'1s activities. Although both the EA-huC'$_1$ and EA-huC'$_{1,4}$ assays were equally valid for detection and measurement of the three C'1 activities, the former method appeared to be the procedure of choice. This conclusion was based on the greater technical simplicity of the EA-huC'$_1$ assay and the theoretical desirability of forming intermediate complexes with purified defined components.

Accordingly, preliminary experiments have been performed on the effect of concentration of two of the C'1 factors on measurement of the third factor in the EA-huC'$_1$ assay. For this purpose, it was first demonstrated that centrifugation and washing of the complex EA-huC'$_1$ was not necessary. The same levels of hemolysis were obtained when human serum diluted in Na_2MgEDTA buffer was added either to the washed complex or directly to the reaction mixture of EA and C'1q, C'1r, and C'1s. In the latter case, after formation of the complex EA-huC'$_1$ at 37°C for 10 minutes, 2.5 ml of 1/62.5 human serum in buffer containing 25.6 ×

10^{-3} M $Na_2MgEDTA$ were added (1/100 serum, 16×10^{-3} M $Na_2MgEDTA$, final concentrations) and incubation was continued at 32°C for 60 minutes.

Using this modified assay procedure, each of the C'1 components was titered in the presence of 1/20, 1/50, 1/100, and 1/200 dilutions of the remaining two C'1 components. In general, little difference was noted in the sensitivity of measurement of any one component using either 1/50 or 1/100 dilutions of the missing components. Diminution of activity was found at lower and higher concentrations. In all cases, high concentrations of the component being

TABLE I

Measurement of C'1s: Effect of Concentration of C'1q and C'1r on Formation of the Complex EA-huC'$_1$

Dilution of C'1s	Dilution of C'1q and C'1r			
	1/20	1/50	1/100	1/200
	*O.D.**	*O.D.**	*O.D.**	*O.D.**
1/20	0.457	0.561	0.589	—
1/40	0.501	0.639	0.619	0.537
1/80	0.588	0.667	0.637	0.552
1/160	0.442	0.634	0.646	0.530
1/320	0.249	0.449	0.547	0.434
1/640	0.093	0.214	0.327	0.213
1/1280	0.043	0.100	0.125	0.092
1/2560	0.018	0.035	0.049	0.017
0	0.007	0.007	0.007	0.010

* Optical density at 541 mμ, corrected for EA control.

titered were inhibitory. The data in Table I for measurement of C'1s are similar to those obtained for analogous measurements of C'1q and C'1r.

These experiments, which provide a basis for assay of C'1q, C'1r, and C'1s, demonstrated very clearly a synergistic effect of these components in formation of the complex EA-huC'$_1$. For example, referring to Table I, 1/50 dilutions of C'1q and C'1r were inactive in the absence of C'1s, while significant formation of the complex EA-huC'$_1$ could be achieved by the addition of a dilution of 1/640 of C'1s. Assuming for C'1s a value of 10 for $E\,\frac{1\text{ per cent}}{1\text{ cm}}$ at 280 mμ, this would correspond to 0.15 μg of protein in the assay system.

It was pointed out in the section on the role of C'1q, C'1r, and C'1s in the formation of the complex EA-huC'$_{1,4}$ from EA-huC'$_4$ that significant levels of lysis with C'1r and C'1s in the absence of C'1q were occasionally observed. This phenomenon has also been noted, again only with respect to C'1r and C'1s, in formation of the complex EA-huC'$_1$, but the occurrence was related to the

method of preparation of EA. If EA made with heated anti-Forssman rabbit serum was washed twice with an equal volume of TBS-gelatin buffer, C'1r and C'1s were completely and consistently inactive in forming the complex EAhuC'$_1$, unless a source of C'1q was added.

C'1 activity as measured in the cold phase of the Donath-Landsteiner reaction: C'1q, C'1r, and C'1s were tested individually and in various combinations for their ability to complete the initial or cold phase of the Donath-Landsteiner reaction. As indicated in Table II, the fractions individually or in combinations of two were inactive, while combination of all three factors restored C'1

TABLE II

Participation of C'1q, C'1r, and C'1s in the Cold Phase of the Donath-Landsteiner Reaction

Fractions in cold phase			Completion of cold phase
C'1q	C'1r	C'1s	Hemolysis with R1
ml	*ml*	*ml*	*O.D., 541 mμ*
—	—	—	0.030
0.10	—	—	0.030
—	0.10	—	0.030
—	—	0.10	0.030
0.10	0.10	—	0.030
—	0.10	0.10	0.030
0.10	—	0.10	0.030
0.10	0.10	0.10	0.450

activity. At higher levels of antibody, a small amount of hemolysis occurred in the presence of C'1r and C'1s, in the absence of C'1q.

In contrast to the hemolytic systems described above, the activities of C'1r and C'1s could be diluted out more rapidly than C'1q. This was determined by serial dilution of each of the components individually while maintaining the other two at constant high levels. Half-maximal hemolysis was obtained at a dilution of 1/64 with C'1q, 1/6 with C'1r, and 1/4 with C'1s. The markedly diminished sensitivity of this immune human hemolytic system in comparison with the hemolytic systems described above is apparent.

C. Properties of Chromatographic Fractions as a Source of C'1 Esterase.— Individual chromatographic fractions, recalcified and adjusted to ionic strength 0.15 by dilution, were tested for their ability to generate C'1 esterase. No single fraction or any combination of two fractions was capable of forming C'1 esterase during incubation at 37°C for 90 minutes. However, reaction mixtures containing C'1q, C'1r, and C'1s generated relatively large amounts of this enzyme under these conditions. The activity of C'1q, C'1r, and C'1s

with respect to activation of C'1 esterase is shown in Fig. 4. The chromatogram is the same as Fig. 3. Activity peaks were obtained in a manner conceptually analogous to that described for the measurement of these components in hemolytic systems.

For example, the C'1q activity peak was obtained as follows: C'1s (pool of fractions 98 to 101) was adjusted to ionic strength 0.15 by diluting 1/2.46 with distilled water and sufficient M/80$CaCl_2$ to give a free Ca^{++} concentration of 10^{-3} M. C'1r (pool of fractions 59 to 63) was similarly adjusted with a dilution of 1/1.86. Fractions to be tested for C'1q activity were

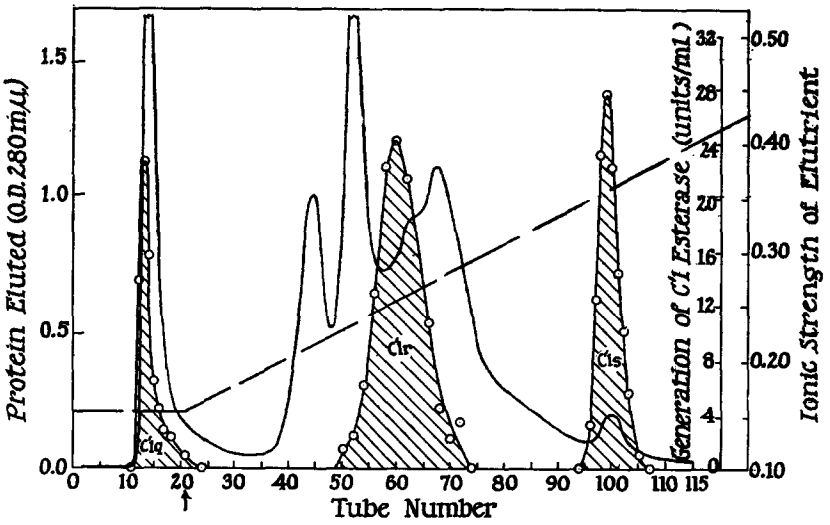

FIG. 4. Chromatographic resolution of C'1 activity of euglobulin fraction into three activities: generation of C'1 esterase; ——, protein eluted; — —, ionic strength of elutrient; cross-hatched areas, C'1 activities. The arrow indicates the start of the salt gradient. Chromatogram same as shown in Fig. 3.

diluted as necessary to achieve ionic strength 0.15 and 10^{-3} M Ca^{++}. The reaction mixtures then consisted of 1.0 ml of C'1s, 0.50 ml of C'1r, and 0.50 ml of the fraction to be tested for C'1q activity. After incubation at 37°C for 30 minutes, 0.375 ml of pH 7.4 phosphate buffer, ionic strength 1.07, was added and the mixtures cooled in ice. By raising the ionic strength to 0.3 in this manner and by lowering the temperature, further activation of C'1 was retarded (13). The additional buffer also was necessary to permit accurate measurement of C'1 esterase by esterolysis. The entire 2.375 ml of reaction mixture was then assayed by addition at 37°C of 0.125 ml of 1 M *N*-acetyl-L-tyrosine ethyl ester in methyl cellosolve and performing microformol titrations with 0.05 N NaOH on 1.0 ml aliquots removed at 0 and 15 minutes. The results are expressed as units of C'1 esterase per 1.0 ml aliquot, where 1 unit corresponds to the liberation of 0.5 micromole of H^+ (32, 33).

Generation of C'1 esterase occurred only when fractions from the pregradient protein peak were added to C'1r and C'1s. The activity peak shown on the left of Fig. 4 corresponded to the C'1q peak shown in Fig. 3 for EA-huC'$_1$ formation with the identical fractions.

The C'1r peak in the enzyme activation assay was obtained by incubating 1.0 ml of C'1s,

0.50 ml of C'1q (pool of fractions 13 to 15), and 0.50 ml of the remaining fractions of the chromatogram under the conditions already described. Finally C'1s was measured in the enzyme activation assay by incubating 0.50 ml of C'1q, 0.50 ml of C'1r, and 1.0 ml of the fraction to be tested for C'1s activity under the same conditions. As in the case of C'1q, there was excellent correlation between C'1r and C'1s as measured either in the esterolytic or hemolytic assays (compare Figures 2, 3, and 4).

Enzyme generation occurred as a function of time at 37°C and was half-maximal at 8 minutes and essentially complete at 15 minutes, using peak fractions of C'1q, C'1r, and C'1s. Activation was not inhibited by soy bean trypsin inhibitor at a final concentration of 5 mg/ml.

D. Summary of Properties of Chromatographic Fractions as a Source of C'1.—Chromatography of the euglobulin fraction resulted in the resolution of C'1 activity into three discrete activities, designated C'1q, C'1r, and C'1s. The participation of all three of these factors for reconstitution of C'1 activity was demonstrated by their requirement for: (*a*) formation of the complex EA-huC'$_{1,4}$ from EA-huC'$_4$; (*b*) direct formation of the complex EA-huC'$_1$; (*c*) completion of the cold phase of the Donath-Landsteiner reaction; and (*d*) generation of C'1 esterase during incubation at 37°C.

III. Identification of the Three Chromatographic Activities of C'1

Before investigating the possible relationship of C'1q, C'1r, and C'1s to known components of complement, the possibility was considered that one or more of these activities was an expression of antibody to erythrocytes. However, absorption of each fraction with an equal volume of washed, packed sheep erythrocytes at 1°C for 1 hour did *not* result in significant loss of activity in formation of the complex EA-huC'$_1$.

A. Identity of C'1q with 11S Component.—When individual chromatographic fractions were assayed for 11S component by the method of Müller-Eberhard and Kunkel (8), large amounts were found in the pregradient area of the chromatogram.[3] Fig. 5 shows the correlation between concentrations of protein and of 11S component in the fractions emerging from the column before and immediately after gradient elution. The distribution of 11S component closely paralleled the distribution of C'1q activity as measured with the same fractions in hemolytic systems and in the enzyme activation assay. This correlation is illustrated by comparing Fig. 5 with the corresponding area of Figs. 2 to 4.

The possible identity of C'1q with 11S component was further investigated by testing the activity of purified 11S component as a substitute for C'1q in hemolytic and enzymatic assays for C'1. In all cases, the C'1 activity of mixtures of C'1r and C'1s was reconstituted by addition of purified 11S component. The level of activity was comparable to that achieved with C'1q

[3] This observation was first made by Dr. Floyd Green in this laboratory in 1961.

fractions containing corresponding concentrations of 11S component, as measured with EA and R11S (8). This conclusion is illustrated in Fig. 6 for formation of the complex EA-huC'$_{1,4}$ from EA-huC'$_4$ and is representative of analogous data for formation of the complex EA-huC'$_1$, for completion of the

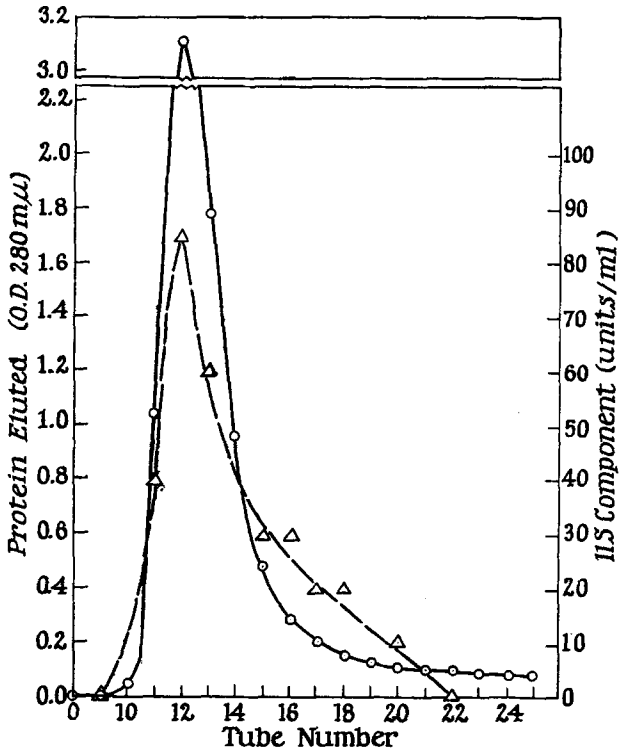

FIG. 5. Identification of C'1q with 11S component: △, 11S component activity; ○, protein eluted in the pre- and immediately postgradient area of the chromatogram.

cold phase of the Donath-Landsteiner reaction, and for generation of C'1 esterase.

Final proof of the identity of the 11S component with C'1q and its lack of identity with C'1r and C'1s was provided by experiments with a euglobulin prepared from R11S. Chromatographic separation of this preparation was performed under the same conditions used for euglobulin fractions from normal human serum. The pregradient fractions from euglobulin devoid of 11S component activity were also devoid of C'1q activity as measured by formation of the complexes EA-huC'$_1$ and EA-huC'$_{1,4}$ and by generation of C'1 esterase.

Nevertheless, C'1r and C'1s activities were present in their usual positions on the chromatogram. Data supporting these conclusions are presented in Table III.

B. C'1r as a New Component of Complement.—Chromatographic fractions of euglobulin prepared from serum were examined for the presence of other components of complement. Significant C'2 activity was not found in the euglobulin fraction and was therefore not sought in column fractions. As

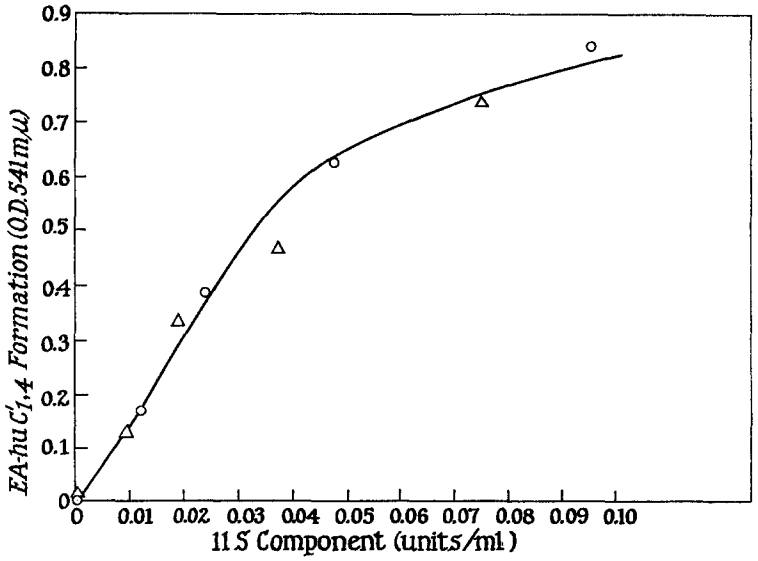

FIG. 6. Identification of C'1q with 11S component: formation of EA-huC'$_{1,4}$ from EA-huC'$_4$, using C'1r, C'1s, and chromatographically separated C'1q, ○; C'1r, C'1s, and 11S component purified with aggregated gamma globulin, △.

shown in Fig. 7, C'3a activity was eluted from the column at a slightly lower ionic strength than C'1r. The C'3a peak was obtained by suspending pellets of the complex EA-huC'$_{1,4,2}$ (5×10^8 cells) in 4.0 ml of 1/4000 dilutions of fresh guinea pig serum, containing 8×10^{-3}M Na_3HEDTA, and 0.1 ml of the fraction to be tested. The mixtures were incubated at 32°C for 60 minutes and the extent of hemolysis determined as usual (29). Small amounts of total C'3 activity could also be detected in the same region of the chromatogram, as measured by restoration of the hemolytic activity of R3.

C'4 activity was eluted from the column at a slightly higher ionic strength than C'1r (Fig. 7). The C'4 peak was obtained by serial dilution assay with EA and R4. In order to increase the sensitivity of detecting C'4, a source of C'3a was added to R4 (0.1 ml of R4 + 0.1 ml of recalcified pool of fractions

51 and 52 to each assay tube). The concentrations of C'4 shown in Fig. 7 are therefore artificially magnified.

It was clear from these experiments that C'1r could not be related to C'2, C'3, or C'4. Furthermore, its participation in reactions requiring C'1 served to define C'1r as part of the activity classically ascribed to C'1. It had already

TABLE III

Comparison of Activities of Chromatographic Fractions of Euglobulin Prepared from Normal Human Serum and R11S

Source of fraction			Formation of complex*		Generation of C'1-esterase‡
C'1q	C'1r	C'1s	EA-huC'$_1$	EA-huC'$_{1,4}$	
			O.D., 541 mμ	*O.D., 541 mμ*	*units/ml*
Serum	Serum	Serum	0.650	1.00	17.0
R11S	Serum	Serum	0.000	—	0
Serum	R11S	Serum	0.520	—	16.1
Serum	Serum	R11S	0.630	—	7.7§
Serum	R11S	R11S	0.680	1.04	7.7§
R11S	R11S	R11S	0.000	0.000	0
11S component	R11S	R11S	0.580	0.950	—

* Complexes were formed as described previously using pellets of EA or EA-huC'$_4$ (5×10^8/ml) and 0.5 ml each of C'1q, C'1r, and C'1s at dilutions of 1/100 for fractions derived from normal serum and 1/10 for fractions derived from R11S. The latter high concentration was selected to compensate for the dilution incurred as a result of chromatographing 1/4 the usual amount of euglobulin on a standard size column. Purified 11S component was substituted for C'1q, using 0.5 ml of a dilution containing 0.04 unit/ml.

‡ Pools of fractions from the C'1q, C'1r, and C'1s areas of the R11S-euglobulin chromatogram only were lyophilized, concentrated 3-fold with respect to the original column fractions, dialyzed *vs.* TBS buffer at 1°C, and used for assay of C'1 esterase generation as described previously. Corresponding fractions from the normal euglobulin were adjusted to ionic strength 0.15 by dilution and used without further concentration.

§ C'1s was concentrated an additional 4-fold by dialysis *vs.* carbowax in order to obtain these significant levels of C'1 esterase activity.

been shown that C'1q was identical with the 11S component and it will be shown below that C'1s is probably identical with C'1 proesterase. On the basis of these considerations, C'1r is proposed as a new component of complement.

C. Identity of C'1s with C'1 Proesterase.—Incubation of euglobulin at 37°C for 15 minutes in the absence of Na_3HEDTA results in generation of C'1 esterase. When such euglobulin is chromatographed under conditions similar to those described above, C'1 esterase activity is found in fractions eluted at ionic strength 0.37 (34). It will be recalled that C'1s is also eluted at this ionic strength. The close correspondence of chromatographic behavior of C'1 esterase and C'1s was confirmed by performing separations, under identical

chromatographic conditions, of a single euglobulin fraction, half of which had been activated to C'1 esterase. C'1 esterase and C'1s were again eluted at ionic strength 0.37. Since C'1s had no esterolytic activity until reacted with C'1q and C'1r and since C'1s and C'1 esterase were chromatographically related, C'1s was identified as C'1 proesterase. This conclusion has been confirmed by the antigenic similarity between C'1s and C'1 esterase, demonstrable by gel diffusion and immunoelectrophoretic analyses with rabbit antisera to human C'1 esterase (35).

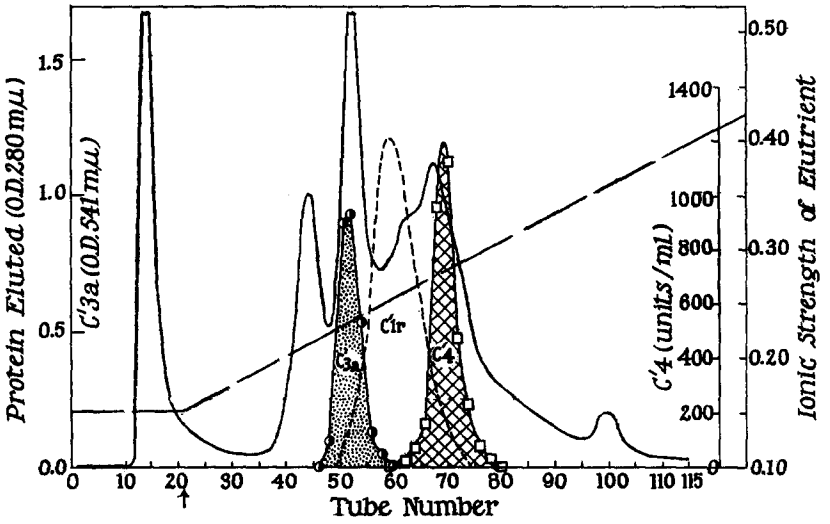

FIG. 7. Lack of identity of C'1r with C'3a and C'4: ——, protein eluted; — —, ionic strength of elutrient. The arrow indicates the start of the salt gradient. Chromatogram same as shown in Figs. 3 and 4.

DISCUSSION

The experiments reported in this paper have shown that the first component of human complement, an activity previously assumed to be a single substance, may be chromatographically resolved into three separate activities. It is of interest to examine earlier attempts at purification of C'1 in terms of lack of recognition of the multiple nature of this component.

Pillemer and coworkers (36, 37) obtained preparations of guinea pig and human C'1 which were relatively highly purified on the basis of electrophoretic and ultracentrifugal criteria. However, more precise methods for the measurement of C'1 were not then available. The assay employed depended upon reconstitution of the hemolytic activity of R1 with a volume of purified fraction equivalent to 1 hemolytic unit in the original serum. Using this method, complete hemolysis was usually achieved, but no information is available on the extent to which the fractions could be further diluted

and still retain hemolytic activity. This point is of particular importance since a recovery of C'1 of only a few per cent would suffice to give complete hemolysis, when tested in the manner described. Indeed, human C'1 recently prepared by the method of Pillemer, Seifter, San Clemente, and Ecker (37) restored hemolytic activity to R1 when the fraction was tested at high concentration. However, the yield obtained in this single trial was less than 1 per cent, as determined by serial dilution assay with R1. The final fraction (1BD) was active at high concentration in forming the complex EA-huC'$_1$ and contained small amounts of C'1q, C'1r, and C'1s. A discard fraction (1BB) was particularly rich in C'1q (11S component). Thus, the C'1 preparation of Pillemer and coworkers probably represented a mixture, in low yield, of the three activities of C'1 described in this report.

A preparation of partially purified human C'1 previously described from this laboratory (14) was highly active as a source of C'1 in various immune systems (13, 31, 38–40). However, this fraction was only 30- to 50-fold purified with respect to serum and contained at least three electrophoretic and ultracentrifugal components. Attempted further purification resulted in large apparent losses of activity, a phenomenon probably referable to the resolution of C'1 into separate activities.

Although considerable progress has been made in the chromatographic separation of other components of complement (12, 41, 42), only one method has been described for purification of C'1 by this technique (12, 26). It is noteworthy, however, that the fraction of guinea pig C'1 so obtained contained approximately 45 per cent of the serum protein. Thus, previous failures to demonstrate the multiple nature of C'1 would appear to have been due to the methods of assay employed and to inadequate degrees of purification.

The three activities of human C'1 described in this paper have been designated C'1q, C'1r, and C'1s, in the order of their elution from DEAE cellulose.[4] C'1q was demonstrated to be identical with the 11S component (8–10), thus establishing this component as part of the activity previously ascribed to C'1. It was not possible to relate C'1r to a known component of complement. These studies have therefore provided evidence for the existence of a previously unrecognized component of complement. On the basis of chromatographic data, C'1s was identified as C'1 proesterase. It has been shown that C'1q, C'1r, and C'1s all participate in various hemolytic reactions requiring C'1. For example, the complex EA-huC'$_1$ could be formed by interaction of the sensitized erythrocyte (EA) with small concentrations of each of the three C'1 activities. Such experiments, in which relatively purified fractions were used and in which the role of 11S component was recognized, stand in contrast to previous studies on formation of the complex EAC'$_1$ (23, 25–27).

C'1q, C'1r, and C'1s could each be measured by serial dilution of one of the activities in the presence of a constant amount of the remaining two. This has been demonstrated in formation of the complex EA-huC'$_{1,4}$ from EA-huC'$_4$ and formation of the

[4] This nomenclature has been provisionally adopted as a means of referring to the three activities of human C'1 which have been recognized in this investigation. The series . . . q, r, s . . . was selected to avoid any connotation of function until such functions are definitively established. The series a, b, c . . . was contraindicated because of previous use of C'1a for "activated C'1" or C'1 esterase (24).

complex EA-huC'$_1$. It has also been shown for completion of the cold phase of the Donath-Landsteiner reaction, employing human erythrocytes and human antibody from patients with paroxysmal cold hemoglobinuria. Of these, formation of the complex EA-huC'$_1$ offers the theoretical and practical advantages of permitting direct interaction of a readily available, defined antigen-antibody complex (EA) with purified components. The sensitivity of the EA-huC'$_1$ assay may be illustrated by comparing the detection of 11S component (C'1q) in this system and in the R11S assay described by Müller-Eberhard and Kunkel (8). The lower limit of sensitivity of the latter assay is 1 unit/ml, using the definition of 1 unit described above, whereas in the former assay concentrations less than 0.01 unit/ml may be measured.

In all of the hemolytic systems investigated, the observation has been made that mixtures of C'1r and C'1s occasionally functioned to a limited extent in the absence of a known source of C'1q. The possibilities have been recognized that this may have been due to contamination of C'1r with C'1q, or to the presence of C'1q derived from rabbit hemolysin. Since C'1r and C'1s were inactive in forming the complex EA-huC'$_1$ when EA suspensions were first washed with TBS-gelatin buffer, the former possibility becomes improbable and the latter more plausible. Investigations on the possible presence of functionally active C'1q in heated anti-Forssman rabbit serum and an adequate explanation of this phenomenon are in progress (43). In this connection it is emphasized that although C'1q, C'1r, and C'1s have all been shown to participate in reactions requiring C'1, no definitive conclusions can be drawn at this time concerning their *absolute* requirement.

It is of particular interest that C'1q, C'1r, and C'1s participated not only in hemolytic reactions requiring C'1 but were also involved in generation of C'1 esterase. Activation of this enzyme could be effected when all three of these activities were incubated together under suitable conditions of concentration, temperature, pH and ionic strength. Thus, the reaction of EA with C'1q, C'1r, and C'1s to form the complex EA-huC'$_1$ was correlated with generation of C'1 esterase by interaction of the three components of C'1 in the absence of antigen-antibody complexes. Biochemical events occurring in the presence of the erythrocyte-antibody complex could, therefore, be duplicated and examined in free solution. This observation provides an important tool for further studies on the mechanism of initiation and on possible methods of inhibition of complement action. Definition of the sequence of action of C'1q, C'1r, and C'1s, prerequisite to such studies, has not yet been achieved, although the hypothesis has already been advanced by Müller-Eberhard and Kunkel (8, 9) and by Hinz and Mollner (44) that C'1q (11S component) may be the first component to function.

A framework is now available for assembling biochemical events from first reaction of an antigen-antibody complex with a component of C'1 up to and including formation of the complex EAC'$_{1,4,2}$ (Table IV). Interaction of the immune complex and the three components of C'1 results in formation of the complex EAC'$_1$ and generation of C'1 esterase. This enzyme in turn reacts with C'4 in a manner which is still biochemically undefined but which is almost certainly related to formation of the complex EAC'$_{1,4}$ (45). C'1 esterase also

interacts with C'2 (14). The functional integrity of the enzyme is required for formation of the complex $EAC'_{1,4,2}$ from $EAC'_{1,4}$ and C'2 (40, 45). Recent observations further suggest that C'2 is enzymatically cleaved by C'1 esterase (46) during formation of the complex $EAC'_{1,4,2}$. Thus, the generation and subsequent action of C'1 esterase appear to occupy central positions in the function of C'1, C'4, and C'2. An analogous biochemical framework for the functioning of the components of C'3 has not yet emerged. It is known, however, that C'1 esterase is not involved in the C'3 steps (40, 45) and the terminal morphologic and physiopathologic events of immune cytolysis have been well described (47–49).

TABLE IV

Correlation of Intermediate Reactions and Biochemical Events in Immune Hemolysis

Reactants and intermediate complexes	Biochemical events
$EA + C'1q + C'1r + C'1s \xrightarrow{Ca^{++}} EAC'_1$	Generation of C'1 esterase
$EAC'_1 + C'4 \longrightarrow EAC'_{1,4}$	Interaction of C'1 esterase with C'4; nature of reaction unknown
$EAC'_{1,4} + C'2 \xrightarrow{Mg^{++}} EAC'_{1,4,2}$	Interaction of C'1 esterase with C'2; enzymatic cleavage of C'2; fulfillment of function of C'1 esterase
$EAC'_{1,4,2} + C'3a + C'3b + C'3c \longrightarrow E^*$	Unknown biochemical role of C'3 components, resulting in production of holes in cell membrane, loss of permeability control, and osmotic lysis
$E^* \longrightarrow$ ghost + hemoglobin	

SUMMARY

A euglobulin fraction of human C'1 has been chromatographically resolved into three distinct activities, designated C'1q, C'1r, and C'1s, in the order of their elution from DEAE cellulose. All three of these activities have been shown to participate in various hemolytic reactions requiring C'1, including the cold phase of the Donath-Landsteiner reaction, and to be necessary for generation of C'1 esterase. C'1q was identical with a previously described serum protein implicated in a very early step of complement action and designated the 11S component on the basis of its sedimentation constant. C'1r could not be related to a known complement activity and has been presented as a new component. C'1s, on the basis of chromatographic evidence, was identified with C'1 proesterase. Methods of assay of these components of C'1 have been presented.

The significance of C'1q, C'1r, and C'1s in generation of C'1 esterase and the central role of this enzyme in reactions involving C'1, C'4, and C'2 have been discussed.

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