

## THE MACROMOLECULAR NATURE OF THE FIRST COMPONENT OF HUMAN COMPLEMENT\*

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The first component of human complement (C'1) has been resolved by DEAE cellulose chromatography into three activities, designated C'1q, C'1r, and C'1s, all of which are required for reconstitution of C'1 activity in several hemolytic systems and for generation of C'1 esterase (1). C'1q was shown to be identical with a previously described serum protein which functions in an early step of complement action and which has been designated the 11S component on the basis of its sedimentation constant (2-5). C'1r was presented as a new component of complement and C'1s was identified as C'1 proesterase, the proenzyme associated with C'1 and capable of activation to C'1 esterase (6-9).

The starting material for resolution of C'1 into these three activities was a euglobulin fraction of human serum (1). The original purpose of the investigation was to isolate the proenzymatic form of C'1. A method was therefore sought for inhibiting the activation of C'1 esterase which occurs as a function of time and temperature when euglobulin preparations are adjusted to physiologic conditions of pH and ionic strength (7). Advantage was taken of the inhibitory effect of the sodium salt of ethylenediaminetetraacetic acid (Na<sub>3</sub>HEDTA)(7), and this reagent was added at a concentration of 10<sup>-3</sup>M to the euglobulin fraction and to all elution buffers employed in chromatography (1). The separation and identification of C'1q, C'1r, and C'1s was thereby made possible.

In further studies, it has been found that C'1q, C'1r, and C'1s undergo an association reaction to form a macromolecule possessing the hemolytic function of C'1. This association can be prevented by Na<sub>3</sub>HEDTA or Na<sub>2</sub>MgEDTA. The macromolecule, once formed, can be dissociated into the known subcomponents of C'1 by the same reagents. Experiments have also been performed which in-

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dicates that C'1 exists in normal human serum as a macromolecule which can be dissociated by Na<sub>3</sub>HEDTA or Na<sub>2</sub>MgEDTA into subcomponents identifiable as C'1q, C'1r, and C'1s. Additional insight has therefore been gained into the nature of human C'1 and the mechanism of inhibition of C'1 activity by EDTA. It is the purpose of this paper to report the experimental evidence for these conclusions.

### Materials and Methods

*Salts of Ethylenediaminetetraacetic Acid (EDTA).*—Reagent grade Na<sub>2</sub>H<sub>2</sub>EDTA was titrated to pH 7.4 at a stock concentration of 0.15 M, resulting in a solution largely in the form of Na<sub>3</sub>HEDTA. Na<sub>2</sub>MgEDTA and Na<sub>2</sub>CaEDTA (Geigy Chemical Corp., Ardsley, New York) were also titrated to pH 7.4 at a stock concentration of 0.15 M.

*Buffer Diluents.*—Triethanolamine-buffered saline (TBS) (10) at pH 7.4, ionic strength 0.15, containing 10<sup>-3</sup> M Ca<sup>++</sup> and no Mg<sup>++</sup>, was employed in all experiments unless stated otherwise. TBS-gelatin buffer consisted of 0.05 per cent gelatin (Knox special intravenous, Knox Gelatine Protein Products, Inc., Camden, New Jersey) in TBS buffer. TBS-gelatin-Na<sub>3</sub>HEDTA, TBS-gelatin-Na<sub>2</sub>MgEDTA, and TBS-gelatin-Na<sub>2</sub>CaEDTA buffers were prepared by addition of the appropriate stock reagent to TBS-gelatin buffer.

*Sensitized Sheep Erythrocytes (EA).*—Sheep blood was collected monthly in an equal volume of Alsever's solution and stored at 4°C. The red cells were washed, standardized spectrophotometrically to contain 10<sup>9</sup>/ml, and sensitized with an equal volume of anti-Forsman rabbit serum, as described by Mayer (11). Following sensitization, the cells were washed twice with equal volumes of TBS-gelatin buffer and restandardized to a concentration of 5 × 10<sup>8</sup>/ml. Complete hemolysis of 1.0 ml of sensitized cells in a volume of 4.0 ml yields an OD at 541 mμ of 1.31.

*Human Serum.*—Human blood without anticoagulant was allowed to clot at room temperature, stored overnight at 5°C for maximal clot retraction, and separated by centrifugation in the cold. Pools from 10 to 100 donors were frozen in aliquots and stored at -70°C.

*Human C'1.*—C'1q, C'1r, and C'1s were prepared by DEAE cellulose chromatography in the presence of Na<sub>3</sub>HEDTA, as described previously (1). Pools of these three activities were dialyzed separately vs. TBS buffer without Ca<sup>++</sup> at 1°C for 16 hours, reducing the Na<sub>3</sub>HEDTA concentration in each to 10<sup>-8</sup> M. The optical density of representative pools of C'1q, C'1r, and C'1s measured at 280 mμ in a 1 cm light path was 2, 0.7, and 0.2, respectively. For purposes of expressing dilutions, these pools were considered undiluted. Aliquots were stored at -70°C assuring their stability for at least 2 months. Portions of each component were thawed daily and maintained at 1°C.

*EAC'1.*—This intermediate complex between human complement and EA, possessing the functional activity of C'1, may be prepared by direct reaction of EA with a source of C'1 deficient in C'4 (1, 12-15). In most of the experiments reported here, the subcomponents of C'1 (C'1q, C'1r, and C'1s) were used to form EAC'1. The concentration of Ca<sup>++</sup> was always 10<sup>-3</sup> M. The activity of this complex was quantified by measuring the extent of hemolysis produced by an appropriate dilution of human serum containing 16 × 10<sup>-3</sup> M Na<sub>2</sub>MgEDTA. The Na<sub>2</sub>MgEDTA chelates Ca<sup>++</sup>, blocking further action of C'1 but permitting reaction of C'2, C'3, and C'4. Thus, the activity of the complex EAC'1 provides a means of measuring C'1 in unknown preparations.

*Assay of C'1 Subcomponents.*—C'1q, C'1r, and C'1s were measured essentially as described previously (1). Pellets of EA containing 5 × 10<sup>8</sup> cells were suspended in 0.5 ml of the sample to be tested for a given subcomponent. Aliquots of 0.5 ml of 1/100 dilutions of the other two subcomponents were then added. The diluent was always TBS-Gelatin buffer containing 10<sup>-3</sup>

$m$   $Ca^{++}$ . After incubation at  $37^{\circ}C$  for 10 minutes, EAC'1 activity was measured as described above.

*Kinetic Technique.*—All kinetic experiments were performed in baths controlled to  $\pm 0.1^{\circ}C$ , using a wrist action shaker for mixing. Reactants were preincubated for 5 minutes at the desired temperature before mixing. The same master dilutions of C'1 subcomponents and human serum in TBS-gelatin- $Na_2MgEDTA$  buffer were used in each experimental series.

*Sucrose Density Gradient Ultracentrifugation.*—A slight modification of the design described by Martin and Ames (16) was used to produce linear sucrose gradients. In each of the experiments reported here, 2.3 ml of 40 per cent (w/v) sucrose in TBS buffer was placed in the mixing chamber and 2.3 ml of 10 per cent (w/v) sucrose in TBS buffer was placed in the adjacent chamber. After the valve between the two chambers was opened, connection between the mixing chamber and the outflow tube was established, allowing the gradient to form under gravity flow. Emptying time was approximately 15 minutes. Final volumes of 4.5 ml were collected in lusteroid centrifuge tubes which were stored at  $1^{\circ}C$  for 30 minutes to 2 hours prior to use.

Immediately preceding each run, the solution to be studied was layered on the gradient. A swinging bucket rotor (SW-39) designed for the model L Spinco ultracentrifuge (Beckman Instruments, Inc., Spinco Division, Palo Alto) was used in all experiments. Centrifugation was performed at 35,000 RPM for 16 hours at a chamber temperature of  $7^{\circ}C$ . At the completion of a run, each centrifuge tube was emptied by punching a hole in the bottom with a 26 gauge needle and collecting 30 to 36 samples of 3 drops each. Individual samples, collected in stated volumes of 0.15  $m$  NaCl, were dialyzed overnight at  $1^{\circ}C$  vs. TBS buffer.

For purposes of monitoring the efficiency of protein separation, the sample on one of the three gradients used in each run consisted of a mixture of bovine gamma globulin (BGG) (Cohn fraction II, Armour Laboratories, Chicago) and porcine thyroglobulin (PTG) (Sigma Chemical Co., St. Louis). BGG has a sedimentation constant of about 7S, while PTG provided a marker with reported values of 18.7S to 19.2S (17, 18). Equal volumes of BGG (20 mg/ml) and PTG (5 mg/ml) were mixed and 0.10 ml of this mixture was placed on the sucrose gradient. Following centrifugation the marker gradient was always separated into fractions of 3 drops each and collected in 5.0 ml of 0.15  $m$  NaCl. The optical density of these samples was read at  $210 m\mu$ , using a Zeiss PMQII spectrophotometer. A graph of the optical density of each fraction vs. the position of each fraction in the sucrose gradient demonstrated the relative positions of 7S and 19S proteins. Using these known values, approximate sedimentation values for the materials studied could be calculated.

## EXPERIMENTAL

### *I. Formation of Intermediate Complexes between Sensitized Erythrocytes and Subcomponents of C'1.*—

(a) *Formation of the intermediate complex EAC'1q:* The demonstration that C'1 is composed of three subcomponents (1) suggested that formation of the complex EAC'1 might involve a sequential reaction between sensitized cells (EA) and C'1q, C'1r, and C'1s. Since Müller-Eberhard (4) and Hinz and Mollner (5) had already shown that the 11S component (C'1q) may react with EA to form the intermediate complex EA-11S, experiments were first performed to investigate the reaction between EA and C'1q.

Pellets of EA containing  $5 \times 10^8$  cells were suspended in 1.0 ml of various dilutions of C'1q containing  $10^{-3}$   $m$   $Ca^{++}$ . The mixtures were incubated at  $37^{\circ}C$  for 10 minutes, centrifuged, washed with 5.0 ml of TBS-gelatin buffer at  $37^{\circ}C$ , and the pellets suspended in 0.5

ml of C'1r and 0.5 ml of C'1s, each at 1/100 dilutions containing  $10^{-3}$  M  $\text{Ca}^{++}$ . After incubation at 37°C for 10 minutes, 3.0 ml of 1/75 human serum in buffer containing  $2.13 \times 10^{-2}$  M  $\text{Na}_2\text{MgEDTA}$  and  $6.6 \times 10^{-4}$  M  $\text{Mg}^{++}$  were added to each tube and incubation was continued at 37°C for 60 minutes. Formation of the complex EAC'1 was quantified by the extent of hemolysis as measured by optical density at 541  $\mu$ .

The extent of formation of the complex EAC'1 was a linear function of the concentration of C'1q. However, the results were highly variable. Using volumetric technique, the same experimental conditions, and the same reagents, the extent of formation of the complex EAC'1q varied as much as 100 per cent from one experiment to the next. Furthermore, the efficiency with which C'1q reacted with EA to form the complex EAC'1q was quite low. For example, employing the two step technique outlined above, average hemolysis of about 23 per cent (OD 0.30) was observed using 1/8 dilutions of C'1q and no hemolysis was obtained with 1/64 or greater dilutions of C'1q. By comparison, 53 per cent hemolysis occurred when 1/100 dilutions of C'1q were used simultaneously with C'1r and C'1s and the EAC'1 complex was washed as in the two step procedure. The efficiency of formation of the complex EAC'1q could not be improved significantly by variations of  $\text{Ca}^{++}$  concentration or time and temperature of incubation of EA with C'1q. Thus, these experiments demonstrated the feasibility of forming the complex EAC'1q, in agreement with earlier observations (4, 5). They indicated, however, that this intermediate was difficult to prepare and that unusually high concentrations of C'1q were required.

(b) *Attempts to form other intermediate complexes between EA and C'1 subcomponents:* Formation of the complex EAC'1 was investigated further by incubating the complex EAC'1q with either C'1r or C'1s at 37°C for 10 minutes, washing with 5.0 ml of TBS-gelatin buffer at 37°C, and then incubating at 37°C for 10 minutes with the missing subcomponent. No formation of the complex EAC'1 was observed under these conditions over a wide range of concentrations of C'1r and C'1s, although simultaneous addition of C'1r and C'1s, as discussed above, resulted in some activity. It was also not possible to form EAC'1 by incubating EA with either C'1r or C'1s and then following with the missing two subcomponents. Thus, although EAC'1q could be prepared with high concentrations of C'1q, attempts to form the complex EAC'1r, EAC'1s, EAC'1q, r, or EAC'1q, s were uniformly unsuccessful. Since sequential formation of EAC'1 from EA and C'1q, C'1r, and C'1s could not be achieved, the possibility was considered that it was necessary for at least two of these subcomponents to react with each other in the fluid phase to form a product capable of combining with EA. This hypothesis was investigated by studying the kinetics of the reaction between EA and mixtures of C'1q, C'1r and C'1s, leading to formation of the complex EAC'1.

## II. Kinetics of Formation of the Complex EAC'1.—

(a) *Effect of time:* The rate of formation of the complex EAC'1 was measured

as a function of time at 33°C, pH 7.4, ionic strength 0.15, using 1/200 dilutions of each C'1 subcomponent in the final reaction mixture.

Master dilutions of C'1q, C'1r, and C'1s at concentrations of 1/10 were each prepared volumetrically in TBS-gelatin buffer containing a final Ca<sup>++</sup> concentration of 10<sup>-3</sup> M and aliquots were preincubated at 33°C for 5 minutes. At zero time, 1.0 ml of each component was added simultaneously to 17 ml of EA (5.88 × 10<sup>8</sup>/ml) suspended in TBS-gelatin buffer containing 10<sup>-3</sup> M Ca<sup>++</sup>. Aliquots of 1.0 ml were withdrawn at frequent accurately recorded

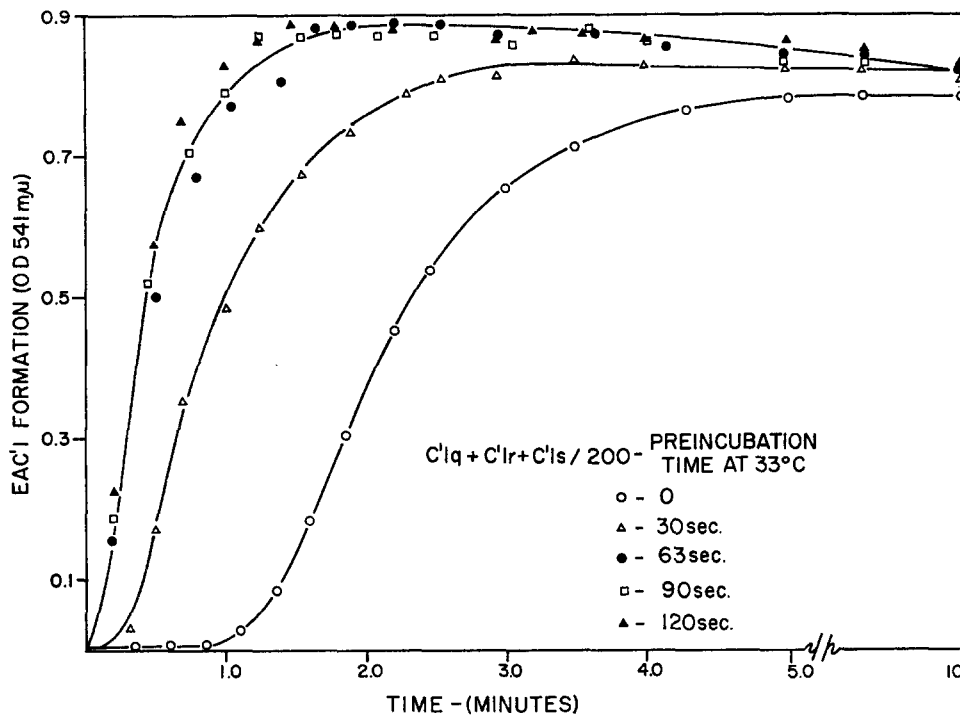


FIG. 1. Effect of time of preincubation of mixtures of C'1q, C'1r, and C'1s on the rate of formation of EAC'1.

intervals of time and placed in 3.0 ml of warm 1/75 human serum in TBS-gelatin buffer containing 2.13 × 10<sup>-2</sup> M Na<sub>2</sub>MgEDTA and 6.6 × 10<sup>-4</sup> M Mg<sup>++</sup>. Formation of the complex EAC'1 was measured by the extent of hemolysis during incubation at 33°C for 60 minutes.

The rate of formation of the complex EAC'1, following the simultaneous addition of separate aliquots of C'1q, C'1r, and C'1s to EA, is shown in the right-hand curve of Fig. 1. It will be noted that formation of the complex EAC'1 was not detected during the 1st minute, reached a maximum within 5 minutes, and remained essentially unchanged for the duration of the experiment.

The effect of time upon formation of the complex EAC'1 was further investi-

gated using the same experimental conditions, except that C'1q, C'1r, and C'1s were mixed at 33°C and incubated for various periods of time before addition of 3.0 ml to 17 ml of EA. As shown in the middle curve of Fig. 1, when the subcomponents of C'1 were incubated together at 33°C for 30 seconds, formation of EAC'1 occurred earlier and more rapidly than when the same concentrations of reactants were added separately and simultaneously. When C'1q,

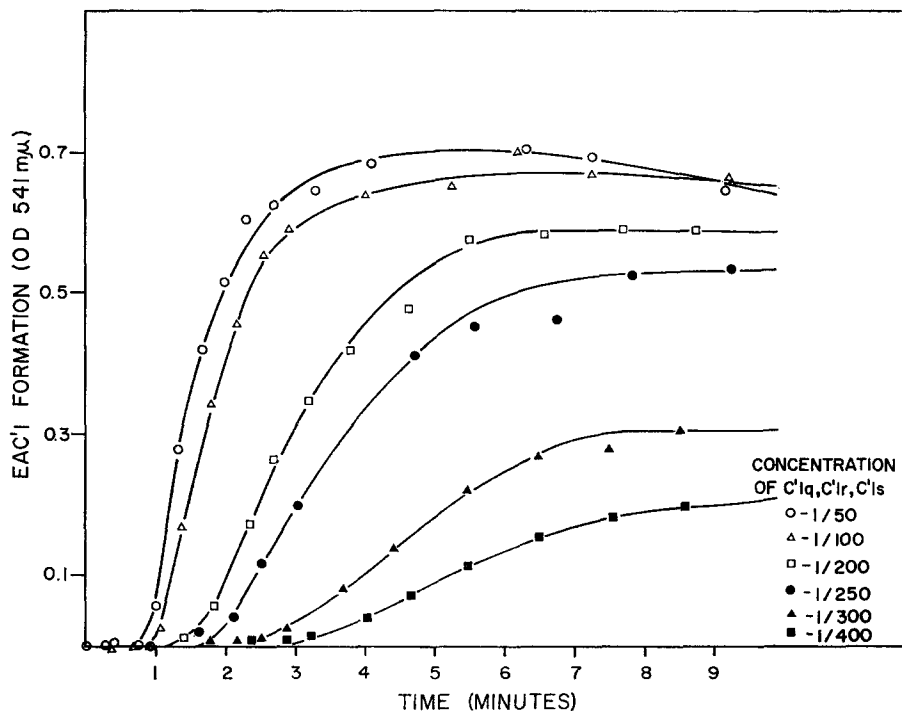


FIG. 2. Effect of concentration of C'1q, C'1r, and C'1s on the rate of formation of EAC'1.

C'1r, and C'1s were incubated together at 33°C for 63, 90, or 120 seconds, formation of EAC'1 was a nearly linear function of time for the first 0.3 minute and reached a maximum at 1.5 minutes (left-hand curve, Fig. 1). Thus, incubation of a mixture of the C'1 subcomponents prior to their addition to EA resulted in reduction or abolition of the time lag for formation of EAC'1 and a decreased time for maximal reaction.

This experiment was consistent with the hypothesis that a reaction between C'1 subcomponents was occurring in the fluid phase and that a product of this reaction was participating in the formation of EAC'1. Further kinetic experiments were performed in which combinations of the two subcomponents were mixed and preincubated for various periods of time before simultaneous addi-

tion to EA of the mixture and the missing subcomponent. In all cases, the time lag and rate of formation of EAC'1 were about the same as in control determinations in which all three subcomponents were added separately and simultaneously. Thus, the kinetic data suggested a reaction in the fluid phase among all three C'1 subcomponents, although the possibility could not be entirely excluded on this basis that only two subcomponents were involved.

(b) *Effect of concentration:* The rate of formation of the complex EAC'1 was measured as a function of concentration of C'1 subcomponents at 32°C. Using the technique described above, formation of EAC'1 was measured following the separate and simultaneous addition to EA of C'1q, C'1r, and C'1s over a final range of dilutions of 1/50 to 1/400 of each subcomponent.

As shown in Fig. 2, the rate and extent of formation of the complex EAC'1 increased as the concentrations of the subcomponents of C'1 were increased. Furthermore, there was a progressive shortening of the time lag for formation of EAC'1 with increasing concentrations of C'1q, C'1r, and C'1s. Other experiments have demonstrated the dependence of the time lag, rate, and extent of formation of the complex EAC'1 upon the concentration of each subcomponent of C'1. For example, when C'1q and C'1r at 1/50 dilutions and C'1s at a 1/200 dilution were mixed separately and simultaneously with EA, the rate and extent of formation of the complex EAC'1 were nearly the same as that found when using each component at a dilution of 1/200.

(c) *Effect of temperature:* Since the time required for formation of the complex EAC'1 was influenced by incubating mixtures of C'1q, C'1r, and C'1s for various periods of time before their addition to EA, this phenomenon was investigated as a function of temperature.

For example, 1.0 ml each of 1/10 dilutions of C'1q, C'1r, and C'1s in TBS-gelatin buffer containing  $10^{-3}$  M  $\text{Ca}^{++}$  were cooled to 0°C and then added separately and simultaneously to 17 ml of EA at 33°C. The time lag for formation of EAC'1 was measured and compared with that obtained when the subcomponents were mixed and held at 0°C for various periods of time before addition to EA. The shortest period of preincubation of the mixture of C'1q, C'1r and C'1s which resulted in minimal or absent time lag was taken as the end-point. By this criterion, the reaction between C'1 subcomponents in the fluid phase was complete in 60 minutes at 0°C, 10 minutes at 15°C, 1 minute at 33°C, and 0.75 minute at 37°C.

The energy of activation for the reaction of C'1 subcomponents in the fluid phase was calculated using the Arrhenius equation,  $V = ze^{-\mu/RT}$  where  $V$  = the velocity of the reaction,  $z$  = a constant,  $e$  = the base of natural logarithms,  $T$  = absolute temperature,  $R$  = the gas constant, and  $\mu$  = the energy of activation (19). The velocity of the reaction at each temperature studied was defined as  $1/t \times 10^2$ , in which  $t$  = the minimal preincubation time (minutes) of the C'1q, C'1r, and C'1s mixture required for elimination of the initial delay in the rate of formation of the complex EAC'1. Since  $t$  was difficult to determine with precision, the possible range of velocity was also calculated from the family of rate

curves obtained at each temperature. A plot of the logarithm of velocity *vs.* the reciprocal of the absolute temperature gave a straight line (Fig. 3) over the temperature range studied. From the slope of this line, a value of 21,000 calories per mol was calculated as the energy of activation, to be compared with previously obtained values of 31,000 calories per mol for activation of C'1 to C'1

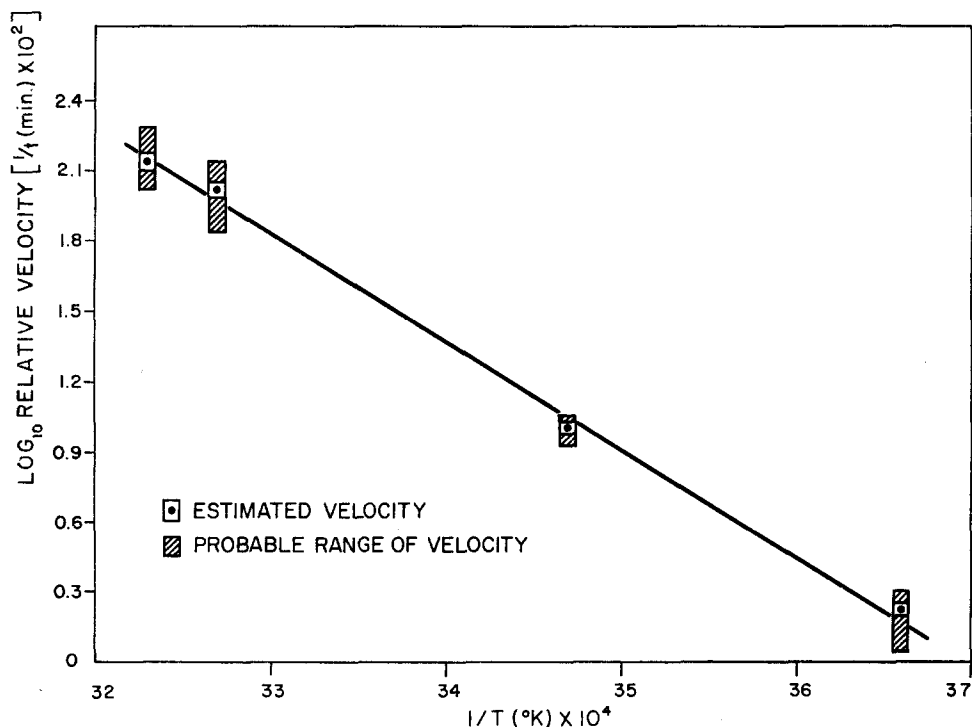


FIG. 3. Arrhenius plot of the rate of interaction of C'1 subcomponents as a function of temperature.

esterase (7) and 10,400 calories per mole for esterolysis of *N*-acetyl-L-tyrosine ethyl ester by a highly purified preparation of C'1 esterase (9).

(d) *Effect of Ca<sup>++</sup> and salts of EDTA:* The participation of Ca<sup>++</sup> in formation of the complex EAC'1 has been well established (11). Furthermore, C'1 in normal human serum can be completely inactivated by treatment with Na<sub>3</sub>HEDTA (20) and the original procedure for resolution of C'1 subcomponents involved the use of Na<sub>3</sub>HEDTA (1). Since preincubation of mixtures of C'1q, C'1r, and C'1s accelerated the rate of formation of the complex EAC'1, it was of interest to investigate the effect of Ca<sup>++</sup> and EDTA on the interaction of the subcomponents of C'1.



On the basis of preliminary investigations, a free  $\text{Ca}^{++}$  concentration of  $10^{-3}$  M was used in all experiments at the step involving actual formation of the complex EAC'1; *i.e.*, in the final reaction mixture consisting of EA and the three subcomponents of C'1.

The effect of  $\text{Ca}^{++}$  on the interaction of C'1 subcomponents was then investigated.

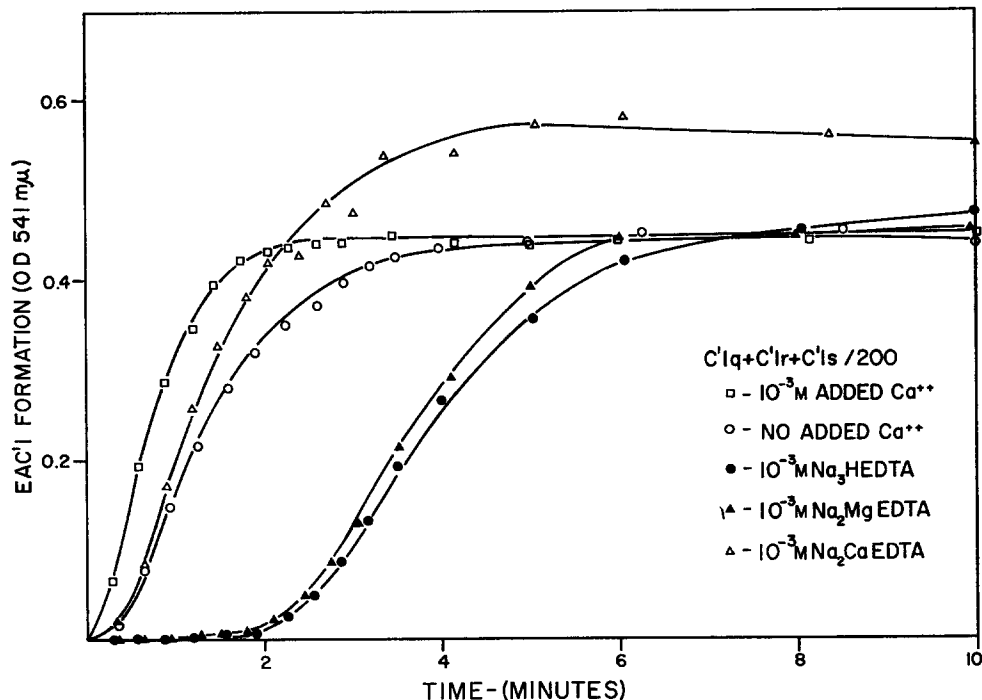


FIG. 4. Effect of  $\text{Ca}^{++}$  and salts of EDTA on the interaction of C'1 subcomponents, as measured by relative rates of formation of EAC'1.

Master dilutions of 1/50 C'1q, C'1r, and C'1s were each prepared volumetrically in TBS-gelatin buffer containing a final  $\text{Ca}^{++}$  concentration of  $10^{-3}$  M. At zero time, a flask was simultaneously charged with 10 ml aliquots of each component. After incubation at  $32^\circ\text{C}$  for 5 minutes, 15 ml of the reaction mixture was added to 5 ml of EA ( $2 \times 10^9/\text{ml}$ ) at  $32^\circ\text{C}$ . Aliquots of 1.0 ml were withdrawn at frequent intervals and tested for the extent of formation of the complex EAC'1. A companion experiment was performed exactly as described above, except that the C'1 subcomponents were diluted in TBS-gelatin buffer without added  $\text{Ca}^{++}$ .

As shown in Fig. 4, the absence of added  $\text{Ca}^{++}$  during preincubation of C'1 subcomponents resulted in a slight time lag in the onset of formation of the complex EAC'1. However, the delay was small compared with that observed following the simultaneous addition of separate aliquots of C'1q, C'1r, and C'1s to EA

(see Fig. 1). In the presence of  $10^{-3}$  M  $\text{Ca}^{++}$  during preincubation of subcomponents, EAC'1 formation occurred essentially without time lag.

The effect of three salts of EDTA on the interaction of C'1 subcomponents was studied using the same technique.

In this case, 1/50 master dilutions of each subcomponent of C'1 were prepared in TBS-gelatin buffer containing  $10^{-3}$  M EDTA and incubated at  $32^\circ\text{C}$  for 5 minutes before addition to EA. The EA suspension contained sufficient  $\text{Ca}^{++}$  to provide a final calculated free  $\text{Ca}^{++}$  concentration of  $10^{-3}$  M.

As further shown in Fig. 4, a time lag in the formation of the complex EAC'1 was found when C'1q, C'1r, and C'1s were preincubated together in the presence of  $10^{-3}$  M  $\text{Na}_3\text{HEDTA}$  or  $\text{Na}_2\text{MgEDTA}$ , although the final extent of reaction was not affected. A time lag was observed with either of these salts of EDTA at concentrations as low as  $10^{-5}$  M but not at  $10^{-6}$  M. When the three subcomponents of C'1 were preincubated with  $10^{-3}$  M  $\text{Na}_2\text{CaEDTA}$ , formation of the complex EAC'1 was only minimally delayed and, for unexplained reasons, the final extent of reaction was increased. The similarity of effect of  $\text{Na}_3\text{HEDTA}$  and  $\text{Na}_2\text{MgEDTA}$  and the marked difference with  $\text{Na}_2\text{CaEDTA}$  were compatible with a requirement for  $\text{Ca}^{++}$  in the interaction of C'1 subcomponents.

In a second group of experiments, the effect of these salts of EDTA on preincubated mixtures of C'1 subcomponents was investigated. Master dilutions of 1/25 C'1q, C'1r, and C'1s were each prepared volumetrically in TBS-gelatin buffer without  $\text{Ca}^{++}$ . At zero time, a flask was simultaneously charged with 5.0 ml aliquots of each subcomponent. After incubation at  $32^\circ\text{C}$  for 5 minutes, 15 ml of  $2 \times 10^{-3}$  M EDTA in TBS-gelatin buffer was added to the reaction flask. Following an additional 5 minutes incubation period at  $32^\circ\text{C}$ , 15 ml of this reaction mixture was mixed with 5.0 ml of EA ( $2 \times 10^9/\text{ml}$ ) and the extent of formation of the complex EAC'1 was determined at frequent intervals. In control experiments, 15 ml aliquots of TBS-gelatin buffer without  $\text{Ca}^{++}$  were substituted for EDTA in the C'1 reaction mixture.

The results were essentially identical to those shown in Fig. 4. Preincubation of C'1q, C'1r, and C'1s in the absence of added  $\text{Ca}^{++}$  yielded a product which reacted with EA to form EAC'1 without appreciable time lag. Treatment of this product with either  $\text{Na}_3\text{HEDTA}$  or  $\text{Na}_2\text{MgEDTA}$ , but not with  $\text{Na}_2\text{CaEDTA}$ , restored the time lag observed when C'1 subcomponents were added separately and simultaneously to EA. In the case of the preformed product of interaction of the C'1 subcomponents, the minimal effective concentration of  $\text{Na}_3\text{HEDTA}$  or  $\text{Na}_2\text{MgEDTA}$  was  $10^{-4}$  M.

*III. Ultracentrifugal Studies on C'1.*— One possible synthesis of the preceding experiments and of previous investigations (1) was provided by the following hypotheses: (a) C'1q, C'1r, and C'1s react with each other in the fluid phase to form a macromolecular complex capable of forming EAC'1 by direct reaction with EA; (b) association of this macromolecule can be prevented by  $\text{Na}_3\text{HEDTA}$  or  $\text{Na}_2\text{MgEDTA}$ ; (c) once formed, the macromolecule can be

dissociated by  $\text{Na}_3\text{HEDTA}$  or  $\text{Na}_2\text{MgEDTA}$ ; (d) C'1 in normal human serum exists as a macromolecular complex, composed at least in part of C'1q, C'1r, and C'1s, and dissociable by  $\text{Na}_3\text{HEDTA}$  or  $\text{Na}_2\text{MgEDTA}$ . Sucrose density gradient ultracentrifugation of C'1 subcomponents and of normal human serum, in the presence and absence of EDTA, appeared to provide the most incisive approach to the testing of these hypotheses.

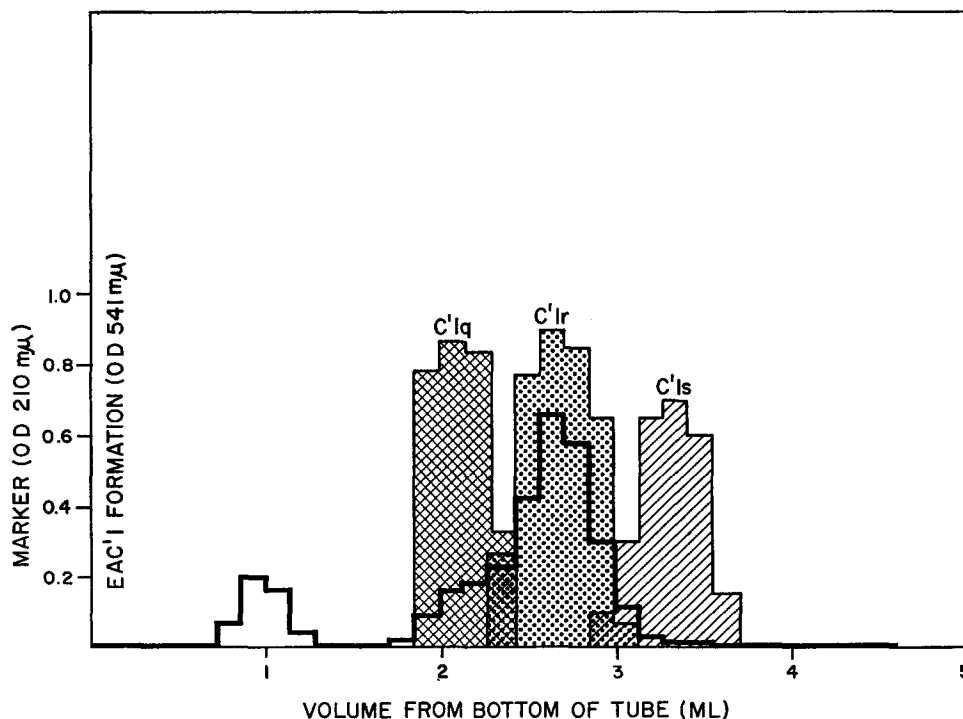


FIG. 5. Ultracentrifugal behavior of individual subcomponents of C'1 in sucrose density gradients: a composite plot of separate determinations on each subcomponent in the presence of  $\text{Na}_3\text{HEDTA}$ . The dark line refers to the distribution of porcine thyroglobulin (left) and bovine gamma globulin (right).

(a) *Sedimentation of individual subcomponents of C'1:* The sedimentation behavior of individual subcomponents of C'1 was initially investigated in the presence of  $\text{Na}_3\text{HEDTA}$ .

Undiluted pools of each subcomponent were adjusted to a final concentration of  $10^{-8}$  M  $\text{Na}_3\text{HEDTA}$  and 0.15 ml was layered on a sucrose gradient containing  $10^{-8}$  M  $\text{Na}_3\text{HEDTA}$ . After centrifugation for 16 hours at 35,000 RPM, each gradient was separated into fractions by collecting 3 drops in 3.0 ml of 0.15 M NaCl. The fractions were dialyzed *vs.* TBS buffer and tested for subcomponent activity, as described in Materials and Methods.

Typical centrifugation patterns of C'1q, C'1r, and C'1s are shown in Fig. 5. The dark solid line represents the distribution of the protein markers when centrifuged under the same conditions as the subcomponents of C'1. Porcine thyroglobulin (PTG), shown as the first peak on the left of Fig. 5, was found 1.0 ml from the bottom of the gradient, whereas bovine gamma globulin (BGG) was 2.67 ml from the bottom. Approximate sedimentation values for the subcomponents of C'1 were obtained by comparing their positions within the sucrose gradient to that of BGG which has a known sedimentation constant of 7S. The estimated values for the subcomponents of C'1 were 11S for C'1q, 7S for C'1r, and 4S for C'1s. These values may be compared with reported sedimentation coefficients of 11.1S and 10.5S for C'1q (2, 3), and about 4S for C'1 esterase (9).

Further experiments showed that the sedimentation rate of C'1q and C'1r was not altered in the presence of  $10^{-3}$  M  $\text{Ca}^{++}$ . However, the apparent sedimentation rate of C'1s was slightly increased to about 5S, an observation for which an explanation is not available.

(b) *Sedimentation of mixtures of subcomponents of C'1*: If interaction of C'1q, C'1r, and C'1s in the fluid phase in fact leads to formation of a macromolecular complex, this molecule should be heavier than any of its component parts.

Therefore, equal volumes of undiluted pools of C'1q, C'1r, and C'1s were adjusted to a final  $\text{Ca}^{++}$  concentration of  $10^{-3}$  M and incubated together for 5 minutes at 33°C. An aliquot of 0.15 ml of this mixture was layered on a sucrose gradient containing  $10^{-3}$  M  $\text{Ca}^{++}$ . The sucrose gradient was centrifuged and fractionated as described above. Individual fractions were dialyzed and tested for their ability to react with EA by themselves to form directly the complex EAC'1.

A typical centrifugation pattern is shown in Fig. 6. Total C'1 activity (macromolecular C'1) was found in fractions between 1.0 ml and 1.7 ml from the bottom of the sucrose gradient but was not detectable in the remaining fractions. An estimated sedimentation value of 18S was obtained by comparison with PTG which has a sedimentation coefficient of approximately 19S. Under the conditions of these experiments, small amounts of free C'1q, C'1r, and C'1s were occasionally found in lighter fractions devoid of total C'1 activity, suggesting either incomplete utilization of the subcomponents of C'1 or an equilibrium state between macromolecular C'1 and its dissociation products.

The effect of  $\text{Na}_3\text{HEDTA}$  upon the formation of macromolecular C'1 was investigated in a companion experiment.

Equal volumes of undiluted pools of C'1q, C'1r, and C'1s were adjusted to a final concentration of  $10^{-3}$  M  $\text{Na}_3\text{HEDTA}$  and incubated together for 5 minutes at 33°C. An aliquot of 0.15 ml was placed on a sucrose gradient containing  $10^{-3}$  M  $\text{Na}_3\text{HEDTA}$ . This gradient was centrifuged, fractionated, and dialyzed as described above.

Formation of the complex EAC'1 was not observed when individual fractions

were tested directly for C'1 activity. Moreover, as shown in Fig. 6, the activities of C'1q, C'1r, and C'1s were detectable in the same positions as individually centrifuged subcomponents. Thus, in the presence of Na<sub>3</sub>HEDTA, no interaction of C'1 subcomponents occurred. It could be shown, however, that each

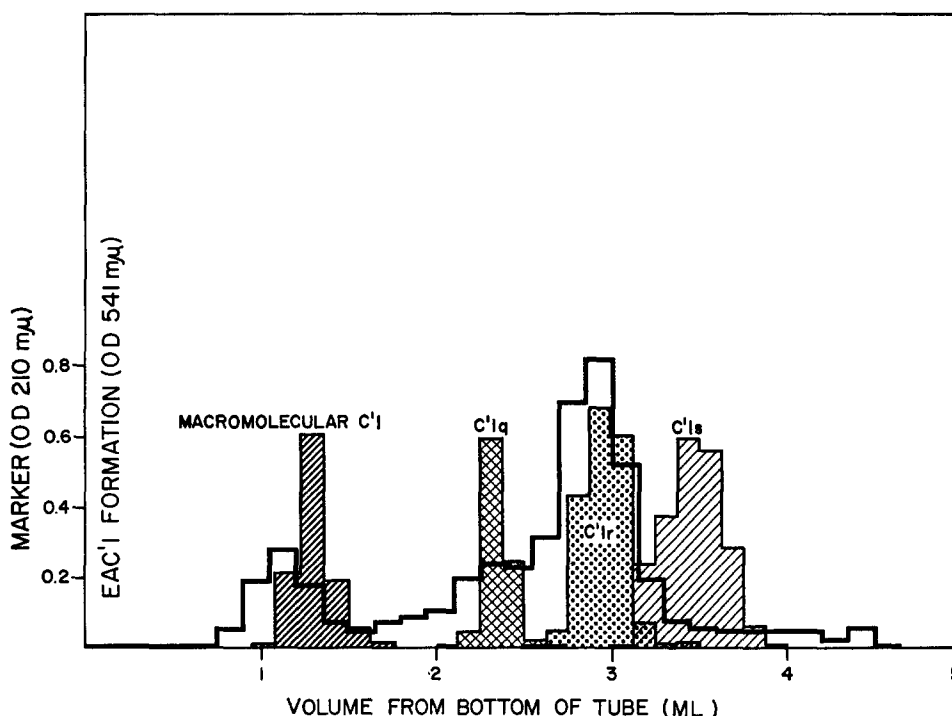


FIG. 6. Ultracentrifugal behavior of mixtures of subcomponents of C'1 in sucrose density gradients: a composite plot of separate gradients simultaneously centrifuged in the absence of Na<sub>3</sub>HEDTA (represented by the peak of macromolecular C'1 on the left) and in the presence of Na<sub>3</sub>HEDTA (represented by the peaks of C'1q, C'1r, and C'1s on the right). The dark line refers to the distribution of porcine thyroglobulin (left) and bovine gamma globulin (right).

component was still active, since recombination and recalcification of the peak fractions of C'1q, C'1r, and C'1s in Fig. 6 produced an activity capable of direct reaction with EA to form EAC'1.

In separate experiments, it was shown that macromolecular C'1, once formed, could be dissociated completely into C'1q, C'1r, and C'1s. Thus, these experiments provided physico-chemical evidence for an association reaction of C'1 subcomponents into a macromolecular complex, prevention of this association by Na<sub>3</sub>HEDTA, and dissociation of the macromolecular complex into C'1 subcomponents by Na<sub>3</sub>HEDTA.

(c) *Sedimentation of C'1 in normal serum*: It was anticipated on the basis of the previous experiments, that C'1 in normal human serum might exist as a macroglobulin dissociable into C'1 subcomponents by  $\text{Na}_3\text{HEDTA}$ . Sucrose gradient fractionation of normal serum in the absence and presence of EDTA was therefore undertaken.

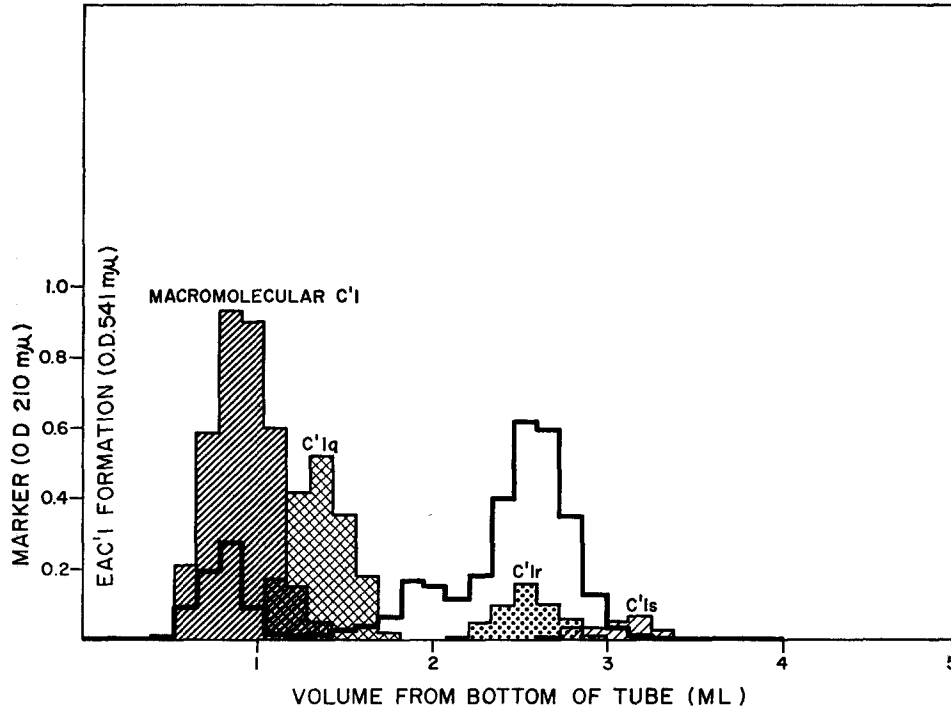


FIG. 7. Ultracentrifugal behavior of C'1 in normal human serum in sucrose density gradients: a composite plot of separate gradients simultaneously centrifuged in the absence of  $\text{Na}_3\text{HEDTA}$  (represented by the peak of macromolecular C'1 on the left) and in the presence of  $\text{Na}_3\text{HEDTA}$  (represented by the peaks of C'1q, C'1r, and C'1s on the right). The dark line refers to the distribution of porcine thyroglobulin (left) and bovine gamma globulin (right).

Serum samples of 0.075 to 0.10 ml were placed on sucrose gradients containing  $10^{-3}$  M  $\text{Ca}^{++}$ , centrifuged, fractionated, and dialyzed in the usual manner. After demonstrating the absence of whole complement, the fractions were tested individually for their ability to form directly the complex EAC'1. As shown in Fig. 7, total C'1 activity was found in the same position within the sucrose gradient as PTG. Free C'1q, C'1r, and C'1s were not detectable. Thus, C'1 activity was associated with the 19S proteins of normal serum. The sedimentation of C'1 in normal serum may be compared with that of macromolecular C'1

prepared from C'1q, C'1r, and C'1s (Fig. 6). Although C'1 in normal serum appeared to be slightly heavier than C'1 prepared from the three subcomponents of C'1, the significance and validity of this observation require further investigation.

In companion experiments, the effect of Na<sub>2</sub>HEDTA on C'1 in normal serum was examined. These experiments were performed exactly as above except that the serum was adjusted to a concentration of 10<sup>-2</sup> M Na<sub>2</sub>HEDTA. The serum (0.075 ml) was again layered on sucrose gradients containing 10<sup>-3</sup> M Na<sub>2</sub>HEDTA. Direct formation of the complex EAC'1 was not observed when any individual fraction (3 drops in 7 ml of 0.15 M NaCl) were tested for total C'1 activity. However, the three subcomponents of C'1 were detected, as shown in Fig. 7, and total C'1 activity was demonstrable by mixing and recalculating aliquots from these areas of the gradient.

Although the activity peaks for C'1r and C'1s were found in their usual positions, C'1q sedimented more rapidly than expected, with an apparent sedimentation constant of 14S to 15S. The reason for this difference in sedimentation behavior of C'1q obtained from dissociation of C'1 in normal serum as compared with C'1q obtained from chromatographic separation of a euglobulin fraction of serum is unknown at the present time but is under investigation.

When a larger volume (0.1 ml) of serum-EDTA solution was subjected to sucrose gradient separation and the fractions collected as 3 drops in 5 ml of 0.15 M NaCl, the same qualitative pattern was obtained. However, in this case, sufficient overlapping of the C'1q, C'1r, and C'1s peaks occurred at a level of 2.5 ml in the centrifuge tube to result in small amounts of total C'1 activity in that region.

The centrifugation pattern obtained with normal serum in the presence of Na<sub>2</sub>HEDTA may be compared with that obtained when C'1q, C'1r, and C'1s were treated with this reagent (Figs. 6 and 7). It is apparent that C'1r and C'1s activities were relatively low as obtained from whole serum. The decreased activity of C'1s may be related to the presence of an inhibitor of C'1 esterase which is normally present in whole serum (21-23) and which has a sedimentation constant of about 3S (24). The inhibitor would, therefore, overlap the position of C'1s in the gradient. This has been confirmed by sucrose density gradient fractionation of mixtures of C'1s and C'1 esterase inhibitor and the demonstration that the amount of C'1s which can be measured in the presence of C'1 esterase inhibitor is markedly reduced. A tenable explanation for the relatively low level of C'1r shown in Fig. 7 is, however, not available at the present time.

#### DISCUSSION

The experiments reported in this paper have demonstrated that the first component of human complement (C'1) is a macromolecular complex composed

of at least three subunits of unequal size. These subunits or subcomponents have been designated C'1q, C'1r, and C'1s and may be prepared from a euglobulin fraction of human serum by chromatography on DEAE cellulose in the presence of Na<sub>2</sub>HEDTA. They are all required for hemolytic C'1 activity and for generation of C'1 esterase (1). Kinetic and ultracentrifugal data indicated, however, that C'1q, C'1r, and C'1s, having apparent sedimentation constants of about 11S, 7S and 4S respectively, acquired hemolytic function by first interacting to form an association product with an apparent sedimentation constant of about 18S. The formation of this macromolecular complex could be prevented by EDTA and the macromolecule, once formed, could be dissociated again into C'1q, C'1r, and C'1s by treatment with EDTA.

These observations suggested that C'1 in normal human serum might also exist as a macromolecule dissociable by EDTA and that the previously reported resolution of C'1 into subcomponents was at least partially referable to such a phenomenon. It was, in fact, shown in the present study that C'1 in human serum sedimented with the macroglobulins as a single protein boundary when sucrose density gradient ultracentrifugations were performed in the absence of EDTA. However, in the presence of EDTA, macromolecular C'1 was no longer demonstrable. Instead, three more slowly sedimenting molecular species with the activities of C'1q, C'1r, and C'1s were detectable and recombination and recalcification of these peaks resulted in reconstitution of total C'1 and direct formation of the intermediate complex EAC'1.

Additional observations, however, indicated possible differences between macromolecular C'1 formed by interaction of isolated C'1 subcomponents and macromolecular C'1 as found in normal human serum. For example, total C'1 activity in serum sedimented slightly but reproducibly faster than the association product of C'1q, C'1r, and C'1s. Furthermore, dissociation of C'1 in serum by EDTA yielded C'1q activity with a larger apparent sedimentation constant than C'1q obtained by column chromatography (1) or by procedures employing aggregated gamma globulin (2, 3). Finally, smaller than expected amounts of C'1r and C'1s activities were obtained by dissociation of C'1 in serum than by dissociation of a comparable amount of macromolecular C'1 formed from isolated C'1 subcomponents. The possibility must therefore be considered that the macromolecular structure of C'1 in serum may be somewhat different from that resulting from association of isolated C'1q, C'1r, and C'1s and that, perhaps, one or more additional subunits of C'1 may yet be recognized. This problem, as well as the relationship of macromolecular C'1 to the known macroglobulins of human serum, is under current investigation.

The only previous report of which we are aware which may be interpreted as evidence for the macroglobulin nature of C'1 is an abstract in 1942 by Eagle and Rosenfeld (25). These investigators ultracentrifuged fresh guinea pig serum at 60,000 RPM for 3 to 6 hours and noted the disappearance of hemolytic comple-



ment activity, a result which could be prevented by centrifugation in the presence of 8.5 per cent NaCl. It was suggested that "mid-piece" (C'1) had a higher sedimentation constant than "end-piece" and that the former was inactivated by ultracentrifugation of serum at isotonic but not hypertonic salt concentrations. Recent experiments in this laboratory have essentially confirmed this phenomenon. Ultracentrifugation of 12 ml of fresh human serum for 20 hours in the cold at 35,000 RPM (No. 40 Spinco rotor; average centrifugal force, 80, 730 g) yielded a gelatinous pellet which, on reconstitution in buffer, was devoid of total hemolytic complement but was highly active in forming the intermediate complex EAC'1. The pellet also contained appreciable quantities of C'1 esterase (16 units/ml on a volume basis equivalent to original serum), as measured by esterolysis of *N*-acetyl-L-tyrosine ethyl ester. Although the supernatant serum fraction still possessed 75 per cent of the hemolytic complement activity of the original serum, mixture of pellet and supernatant fractions in equivalent amounts resulted in destruction of 90 per cent of the residual complement in the supernatant serum. All of these data are consistent with the macroglobulin nature of C'1, its activation to C'1 esterase at isotonic but not hypertonic ionic strengths in the absence of lower molecular weight inhibitors (7, 24), and the ability of C'1 esterase to inactivate C'4 and C'2 in solution (6).

The sodium and magnesium but not the calcium salts of EDTA were active in preventing association of C'1q, C'1r, and C'1s and in dissociating macromolecular C'1 to its subcomponents. This observation indicated a role for  $\text{Ca}^{++}$  in maintenance of macromolecular structure, although the possibility has not been excluded with certainty that  $\text{Na}_3\text{HEDTA}$  or  $\text{Na}_2\text{MgEDTA}$  might be reacting with C'1 without a metal ion intermediary. It is clear, however, that these salts of EDTA have an effect on complement, specifically on C'1, quite apart from their ability to chelate *free*  $\text{Ca}^{++}$ . Although  $\text{Ca}^{++}$  is, in fact, involved in the formation of EAC'1 (11),  $\text{Na}_3\text{HEDTA}$  and  $\text{Na}_2\text{MgEDTA}$  block this step of complement not only by binding a ligand ( $\text{Ca}^{++}$ ) which participates in the interaction of EA and C'1 but also by dissociating macromolecular C'1 into subunits which, until reassociated, are functionally inactive in forming EAC'1. In these terms, recalcification of EDTA-treated serum reverses inhibition by permitting reassociation of C'1 subcomponents to form a macromolecular complex which can react efficiently with EA in the presence of free  $\text{Ca}^{++}$ . This dual function of EDTA provides insight into earlier studies in which it was concluded that  $\text{Ca}^{++}$  was a potentiating ion rather than an absolute requirement for complement activity and that  $\text{Ca}^{++}$  appeared to exert a sparing action on C'1, reducing the absolute concentration of C'1 required for complement fixation (26). In these experiments, depletion of  $\text{Ca}^{++}$  was achieved with the cation exchange resin amberlite IRC-50 (Na cycle) and it was suggested that EDTA might have a toxic effect on complement apart from its capacity to bind free  $\text{Ca}^{++}$ . The concept of free  $\text{Ca}^{++}$  as a potentiating rather than absolute requirement in the

mechanism of action of complement, previously criticized by other workers (11, 27), now merits further investigation in the light of the ability of EDTA to dissociate macromolecular C'1, as reported here. Such studies are in progress.

The effect of EDTA on macromolecular C'1 provides insight both into the mechanism of resolution of C'1 subcomponents described earlier (1) and the first procedures for demonstrating and isolating the 11S component (C'1q) (2, 3). All of these methods have in common the treatment of serum with Na<sub>3</sub>HEDTA, with the original intents of either inhibiting activation of C'1 to C'1 esterase (1) or attachment of C'1 to aggregated gamma globulin (2, 3). It would now appear that EDTA had the then unknown property of dissociating C'1 into subcomponents. One of these subcomponents, C'1q, has been shown to be identical to the 11S component (1), a factor capable of reacting with aggregated gamma globulin or sensitized erythrocytes in the presence or absence of EDTA (2-5). The ability of C'1q to react with EA, at least to a limited extent, has also been confirmed in the present studies. Free C'1q, C'1r, and C'1s activities were not demonstrable in our experiments on ultracentrifugation of human serum in the absence of EDTA, suggesting that the subcomponents are not normally present in high concentration in serum. It is possible that small amounts, undetected by our methods of measurement, may be in equilibrium with a larger concentration of macromolecular C'1. The conclusion may be inferred, however, that the original isolation of the 11S component (2, 3) was largely a consequence of the dissociation of macromolecular C'1 by EDTA.

The existence of subunits is now a well recognized feature of protein structure and a great deal of attention has been given to the subunit structure of enzymes and antibodies (for examples, see references 28 and 29). In the case of enzymes, the generalization holds that the subunits are of equal size. The tryptophane synthetase system of *Escherichia coli* is a notable exception (30) which, as independently suggested by Dr. M. M. Mayer in a private communication, represents the closest known analogy to the macromolecular nature of C'1 reported here. However, the structure of C'1, as currently understood, differs from that of tryptophane synthetase in several important respects: the participation of at least three, rather than two components of unequal size in macromolecular structure; the absence of currently known enzymatic properties of individually tested C'1q, C'1r, or C'1s in contradistinction to the detectable individual enzymatic activities of the A and B components of tryptophane synthetase; the lack of dependence of C'1 esterase activity, once generated, on maintenance of macromolecular structure (9), as compared with loss of complete tryptophane synthetase activity when the A-B complex is dissociated. The C'1 system is further complicated by the problems of binding to antigen-antibody complexes, the role of Ca<sup>++</sup>, and the intervention of inhibitors. Further elucidation of the structure and mechanism of action of C'1 should, therefore, not only contribute more to our understanding of the biochemistry of complement but may also provide an additional model for biologic control of enzymatic activity.

## SUMMARY

Kinetic and ultracentrifugal experiments demonstrated that the previously described subcomponents of human C'1, designated C'1q, C'1r, and C'1s, interacted with each other in liquid phase to form a macromolecule which was then capable of converting sensitized erythrocytes (EA) to the state EAC'1. The apparent sedimentation constants of C'1q, C'1r, and C'1s and of the macromolecular product of their interaction were approximately 11S, 7S, 4S, and 18S respectively. Association of C'1 subcomponents was prevented and dissociation of macromolecular C'1 was effected by Na<sub>3</sub>HEDTA and Na<sub>2</sub>MgEDTA but not by Na<sub>2</sub>CaEDTA. The rate of formation of macromolecular C'1 was a function of concentration of subcomponents and temperature of interaction, with an apparent energy of activation of 21,000 calories per mol.

Ultracentrifugal studies further indicated the macromolecular nature of C'1 in normal human serum. In the absence of EDTA, C'1 sedimented with the serum macroglobulins and C'1 subcomponents were not detected. Conversely, in the presence of EDTA, macromolecular C'1 was not demonstrable and individual C'1 subcomponents could be measured in lighter fractions.

The significance of these observations in relation to previous studies on C'1 subcomponents, the role of Ca<sup>++</sup> in C'1 function, and the subunit structure of enzymes has been discussed.

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*Addendum.*—Since submission of the manuscript, papers by T. Borsos and H. J. Rapp (*J. Immunol.*, 1963, **91**, 851) and T. Borsos, H. J. Rapp, and U. L. Walz (*J. Immunol.*, 1964, **92**, 108) have appeared. Partially purified preparations of guinea pig and human C'1 were described which behaved as single components in forming the intermediate complex EAC'1,4 from EAC'4. No data were given on the ultracentrifugal behavior of these fractions. The isolation procedures employed differed from those in the present studies in at least three ways: manner of preparation of the euglobulin fraction placed on the column, conditions of chromatography, and absence of Na<sub>3</sub>HEDTA in the buffers used for elution. It may be inferred that the procedure of Borsos and Rapp yields an intact form of C'1, perhaps analogous to that obtained in this study by density gradient ultracentrifugation of normal serum, and that dissociation into subcomponents by Na<sub>3</sub>HEDTA might be realizable. These hypotheses are susceptible to direct experimental examination.

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