C1r, Subunit of the First Complement Component: Purification, Properties, and Assay Based on Its Linking Role

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ABSTRACT A method to obtain C1r, a subunit of the first complement component, in a highly purified state has been described for the first time. The stepwise method starts with a neutral euglobulin precipitation, after diethylaminoethyl- and carboxymethyl-cellulose chromatography and a final preparative polyacrylamide electrophoresis step. Such C1r preparations are devoid of C1q and C1s activities and show only one protein band on analytic polyacrylamide electrophoresis. Rabbits injected with this preparation produced antisera showing only one precipitation band. The stability of C1r activity was determined under different conditions, and C1r was found to be labile at 37°C, pH 7–8 and low ionic strength.

The electrophoretic mobility of purified C1r is that of a β-globulin on disc acrylamide electrophoresis and on agarose electrophoresis at pH 8.6. Its molecular weight as estimated by sephadex chromatography is 168,100.

A sensitive hemolytic assay based on the property of C1r to link C1s to C1q and thereby to generate macromolecular $C\bar{1}$ is described. The number of $C\bar{1}$ molecules generated is stoichiometrically related to the concentration of C1r for a fixed C1q and C1s concentration provided that the titration is carried out below the plateau zone. Macromolecular C1 can be separated from free C1s as the former is cell bound.

This method of purification and assay should allow the development of monospecific antisera and further chemical study of C1r.

INTRODUCTION

Purification of individual complement components has been important for study of their biological activities. Production of monospecific antisera has allowed a practical means of measuring complement protein concentrations clinically and has been useful for associating specific proteins with biologic activity (1–3). Antisera have not been described for C1r, C7, and C9.[1] This report describes a method to obtain C1r, a subunit of C1, in a highly purified state.

Cl is the first complement component to combine with antigen-antibody complexes. After this association, C1 is activated from its proenzymatic state (4). Activated C1, $C\bar{1}$, reacts enzymatically with C4 and C2 to form the second enzyme in the complement sequence, $C\bar{4}\bar{2}$, which is capable of cleaving C3 efficiently to produce a capillary permeability factor and chemotactic factor, C3a (5). A C-derived kinin is also produced in association with the activation of these initially reacting enzymes of the complement sequence (6). Understanding the early events in C activation is important as there is evidence that C1 has been activated in systemic lupus erythematosus, acute glomerulonephritis, rheumatoid arthritis, hereditary angioedema, and other miscellaneous diseases.

In 1963 Lepow, Naff, Todd, Pensky, and Hinz discovered that human C1 was a macromolecule composed

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[1] The symbols for complement components used in this paper conform to recommendations agreed upon by a committee sponsored by the World Health Organization. Nomenclature of Complement. 1970. *Immunochemistry* 7: 137. Complement (C) components are designated numerically C1, C2, C3, C4, C5, C6, C7, C8, C9; the subunits of C1 are designated C1q, C1r, C1s; activated components are indicated by placing a bar over the numeral which refers to the active component or subunit; i.e., active C1 = $C\bar{1}$; active C1s = $C\bar{1}s$; convertase = $C\bar{4}\bar{2}$, etc. Since guinea pig components were used except for the C1 subunits which were always from human serum, a superscript indicating the species will be omitted. Cellular intermediates carrying C components are designated EAC . . . followed by the numeral designating the components carried; e.g., EAC14.

of three distinct subunits, C1q, C1r, and C1s (7). Activation of C1s to $C\bar{1}s$ has been studied extensively by Lepow, Wurz, Pillemer, and Ratnoff (8, 9) and by Donaldson (10), and the hypothesis that C1r is an enzyme which activates C1s has been proposed (11). All previous published work on C1r has been performed with relatively crude C1r fractions from a single DEAE-cellulose purification step after euglobulin precipitation. As C1s is chromatographically and electrophoretically heterogeneous (12) and overlaps C1r in its elution pattern from DEAE-cellulose, these studies were done to obtain highly purified C1r.

In addition to purification, a quantitative assay is presented using Borsos and Rapp's method for measurement of purified macromolecular C1. There is good evidence that absolute molecular concentrations of $C\bar{1}$ can be determined (13). Our assay determines the number of $C\bar{1}$ molecules formed when C1r is combined with C1q and C1s. This combination step is similar to the first step in the hemolytic assay described by Lepow et al. (7). We modified that assay in order to use the quantitative $C\bar{1}$ assay and to distinguish activated $C\bar{1}s$ from macromolecular $C\bar{1}$. The assay described in this paper discriminates between the hemolytic function of $C\bar{1}$ and that of $C\bar{1}s$ by the capacity of $C\bar{1}$ molecules to bind to EAC4 cells. $C\bar{1}s$ molecules do not bind and can be washed away (14). Using this assay we have studied the effect of buffers and temperature on the stability of C1r and designed a purification process taking advantage of conditions for maximal stability. Thus a highly purified preparation of C1r and a sensitive assay method are now available and the biological functions of C1r can be further explored in clinical states such as the isolated C1r deficiency discussed by Pickering, Naff, Stroud, Good, and Gewurz (15) in a patient with glomerulonephritis. Also studies of Stroud et al. (2) have shown that the C1 subunits, C1q and C1s, may exist in markedly disparate concentrations. These observations suggest that the synthesis and/or metabolism of these subunits is regulated by independent mechanisms and that their function may be independent.

We anticipate that these methods will serve for the development of monospecific antisera to measure C1r immunochemically and will stimulate further study of these questions.

METHODS

Buffers. The resistance of buffers at 0°C was referred to a standard curve obtained with various molarities of NaCl. The value obtained is expressed as the relative salt concentration (RSC). Veronal buffered saline containing gelatin, Ca^{++}, and Mg^{++}, RSC[2] 0.147 (VBS^{++}) was prepared according to Mayer (16). VBS is the same buffer without gelatin, Ca^{++}, or Mg^{++}. Dextrose Veronal buffered saline ($DGVBS^{++}$), RSC 0.065, was prepared by mixing 4 volumes of VBS^{++} and 6 volumes of 5% dextrose. Both solutions contained 1.0×10^{-3} M Mg^{++}, 1.5×10^{-4} M Ca^{++}, and 0.1 g/100 ml gelatin. EDTA buffer was prepared by mixing 9 volumes of VBS with 1 volume of 0.1 M EDTA at pH 7.4. Buffers at different pH values were made by adding NaCl to a final concentration of 0.02 or 0.15 M to 0.005 M sodium acetate pH 5.0; 0.005 M sodium phosphate pH 6.0, 7.0, and 7.5; or 0.005 M Tris-HCl buffers pH 8.3 and 9.0. The pH of these buffers was measured at the temperature at which they were used.

Complement components. $C\bar{1}$ preparations were obtained by the procedure of Tamura and Nelson (17) abbreviated to a single precipitation. C1q was prepared using the method of Müller-Eberhard (18) and stored at 0°C, pH 5.0, 0.75 RSC phosphate buffer, at a concentration of 990 μg/ml. Highly purified $C\bar{1}s$ was obtained by the method of Nagaki and Stroud (14) using preparative acrylamide electrophoresis instead of Pevikon electrophoresis (12). Guinea pig C2 was prepared by the method of Nelson, Jensen, Gigli, and Tamura (19). Fresh guinea pig serum (Texas Biological, Fort Worth, Tex.) diluted 1/50 in EDTA buffer (C-EDTA) was used as the source of C3-9.

Some of the C1r preparations referred to as "crude C1r" were obtained as by-products of C1s purification after CM- and DEAE-cellulose chromatography. C1r passed through CM cellulose at pH 5.0, RSC 0.1 together with C1s. C1r eluted from DEAE-cellulose before C1s at a RSC of 0.170 with 0.05 M sodium phosphate buffer at pH 7.5, 0.01 M EDTA. Such preparations are contaminated with C4, C3, IgG, and traces of C1s, but do not contain C1q. Crude and purified C1r samples were stored at 0°C or −70°C in EDTA buffer or VBS.

C component titrations. $C\bar{1}$ was titrated using EAC4 as described by Borsos and Rapp (13). When it was necessary to eliminate the unbound $C\bar{1}s$, the $EAC\bar{1}4$ cells were washed twice with $DGVBS^{++}$. $C\bar{1}s$ was assayed hemolytically as described (14). The C1r hemolytic activity was assayed combining C1r with C1q and $C\bar{1}s$ and then reacting the C1 formed with EAC4, C2, and C-EDTA as will be described in detail below. A $C\bar{1}$ site-forming unit ($C\bar{1}$ SFU) of C1r refers to the amount of C1r which, when added to a specific amount of C1q and C1s, produces 1.5×10^8 effective molecules of $C\bar{1}$. The useful concept of a site-forming unit (SFU) is based on the one hit theory of complement activity, and was first applied to C2 (20). It is the amount of a component producing one effective hemolytic site per cell in a system that contains 1.5×10^8 cells/ml. C2 was titrated using the method of Borsos, Rapp, and Mayer (20).

Radial immunodiffusion. $C\bar{1}s$ was assayed by radial immunodiffusion as described (12). C1q content was determined using monospecific antisera prepared by the method of Yonemasu and Stroud,[3] using a modification of the method of Mancini, Carbonara, and Heremans (21).

Immunodiffusion and immunoelectrophoresis. Immunoelectrophoresis was performed using the method of Scheidegger with 1% agarose (Seakem, Bausch & Lomb Inc. Rochester, N. Y.) support medium or 1.3% agar noble Difco lot 462512 (Difco, Inc., Findlay, Ohio). Buffers, unless stated otherwise,

[2] *Abbreviations used in this paper:* RSC, relative salt concentration; VBS, Veronal buffered saline; DGVBS, dextrose Veronal-buffered saline; SFU, site-forming unit; TCA, trichloracetic acid; CM, carboxymethyl.

[3] Yonemasu, K., and R. M. Stroud. 1971. *J. Immunol.* 106.

contained 0.010 M EDTA. The sensitivity of double diffusion in Ouchterlony and immunoelectrophoretic analyses was increased using the method of van Oss and Bronson (22).

Cellulose chromatography. Fibrous DEAE- and CM-cellulose, used for the purification of C1r, were purchased from Whatman Paper Co. (DE-23 and CM-23) and prepared according to their recommendations. DEAE-cellulose was equilibrated with 0.005 M Tris-HCl buffer, pH 8.2–8.3, 0.09 M NaCl (RSC 0.105–0.110) with or without 0.01 M EDTA (EDTA was added to the buffers if the sample to be applied contained C1q). CM-cellulose was equilibrated with 0.02 M acetate buffer pH 5.0, containing 0.025 M NaCl (RSC = 0.030). Nonlinear gradients were used for the elution of the protein fractions, and details of the procedure will be described below.

Disc acrylamide electrophoresis. The method of Davis was used for analytic disc electrophoresis (23). Following the electrophoretic run the gels were fixed 1 hr in 10% trichloracetic acid (TCA) and stained overnight with 0.1% Coomassie Brilliant Blue dye in 10% TCA. Residual dye was removed by repeated washes with 10% TCA. Studies of the sensitivity of this method showed that it was possible to detect 0.005 mg of human serum albumin.

Preparative acrylamide electrophoresis was done at 4°C using gel columns of 1.3 cm diameter, 5 cm running gel height, 1.5 cm stacking gel height, and the same buffers and gel solutions as for analytical runs.

Samples with an absorbance of 0.100–0.300. OD_{280} U/ml[4] and 5% sucrose were applied in volumes of 0.5–1.0 ml on the top of the stacking gel. 2.5% sucrose in running buffer was carefully overlayered to stabilize the sample zone.

Overnight runs for preparation of C1r were performed at 100 v in the cold room at 4°C for 15 hr. The gels were sliced in 2 mm segments and eluted with 0.15 M NaCl at 0°C. All buffers and gels were precooled before the application of C1r to avoid losses of activity.

RESULTS

Stability of C1r. Attempts to purify C1r beyond the initial chromatographic step described by Lepow et al. (7) were difficult due to the instability of this component under certain conditions.

Using crude C1r preparations obtained after CM-cellulose and DEAE-cellulose chromatography of $C\bar{1}$ as a by product of C1s purification, a preliminary study on the stability of crude C1r was made. Dilutions of crude C1r containing 200 $C\bar{1}$ SFU/ml were incubated at 0°C or at 25°C for 24 hr at various ionic strengths and pH 7.5 as shown in Table I. After incubation the preparations were diluted and the residual C1r activity was assayed by adding C1q and $C\bar{1}$s as described below.

The results indicate that some activity was lost even at 0°C when compared to the control sample diluted from the stock C1r solution in $DGVBS^{++}$ and assayed without incubation. However, there was a greater loss of activity in the samples that were held at room temperature. 95% of the original activity was lost in the sample diluted in 0.015 RSC pH 7.4 buffer and held at room temperature for 24 hr.

TABLE I

Effect of Ionic Strength on Activity of C1r

Buffer RSC	$C\bar{1}$ after 24 hr of incubation, pH 7.5	
	0°C	25°C
	SFU/ml	*SFU/ml*
0.015	83	11
0.032	142	23
0.057	133	74
0.050	133	100
0.145	95	65
0.240	91	60

C1r No. 16 was diluted in buffers of different relative salt concentrations (see Methods), pH 7.5, 0.005 M phosphate at a concentration of 200 $C\bar{1}$ SFU/ml, incubated for 24 hr at 25°C or at 0°C, and the residual C1r hemolytic activity was assayed.

The effect of pH variation was explored at 0.02 RSC or 0.15 RSC using the buffers described above. As shown in Table II, portions of C1r containing 36.0 $C\bar{1}$ SFU/ml of C1r were incubated at 37° or 0°C for 1 hr in these buffers. The residual C1r activity was determined after a sufficiently high dilution of the C1r samples in $DGVBS^{++}$ to adjust the pH to 7.4 and the RSC to 0.065. It was found that there was a sharp decrease in activity when the samples were incubated at pH 7–8 and 37°C. This instability of C1r was most pronounced at lower ionic strengths and pH 7.6. At 0°C, however, C1r activity was stable for this time over a wide pH range.

The stability of C1r at pH 7.5 and RSC 0.02 was explored at various concentrations of C1r. A highly

TABLE II

Effect of pH on Activity of C1r

0°C				37°C			
0.15 RSC		0.023 RSC		0.15 RSC		0.023 RSC	
pH	*SFU/ml*	*pH*	*SFU/ml*	*pH*	*SFU/ml*	*pH*	*SFU/ml*
5.15	35.5	5.2	38.8	5.15	20.7	5.2	16.0
6.15	36.36	6.45	35.28	6.15	29.2	6.45	18.4
7.0	36.36			7.0	19.4	7.25	9.2
7.3	30.36	7.25	52.0	7.3	15.72	7.60	8.96
7.75	34.12	7.65	35.28	7.75	9.44	7.65	14.0
8.01	32.04	8.01	37.20	8.01	7.80	8.01	14.0

C1r No. 16 was diluted in buffers of different pH and relative salt concentrations (RSC), at a concentration of 36.0 $C\bar{1}$ SFU/ml. After 1 hr of incubation at either 37°C or 0°C the residual C1r activity was assayed by addition of C1q and C1s.

[4] One optical density unit at 280 mμ (OD_{280} U) is the optical density of a solution at 280 mμ in a 1 cm light path cuvette.

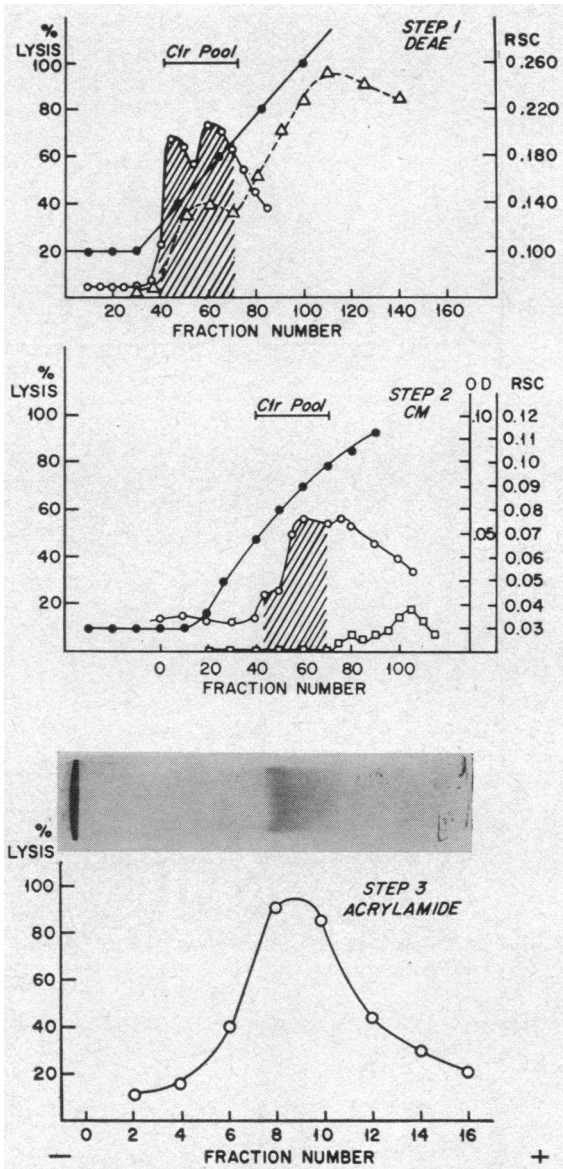

FIGURE 1 Purification of C1r. The activities of C1r (○——○) and of C1s (△——△) were determined by the scanning method described in the text. RSC (●——●) OD_{280} (□——□).

Step 1: DEAE-cellulose; applied neutral euglobulin from human serum. Step 2: CM-cellulose; applied C1r pool from step 1. Step 3: Preparative disc acrylamide electrophoresis of C1r pool from step 2; See text for experimental details.

purified C1r preparation (Fig. 1, post-CM) was used. 0.1% gelatin was added to the solutions to prevent the loss of highly dilute C1r by nonspecific adsorption. Dilutions of C1r containing 4–64 $C\bar{1}$ SFU/ml were made in 0.1% gelatin, 0.020 RSC, and 0.005 M phosphate buffer. One portion of each dilution was incubated at 37°C for 1 hr and the other portion kept at 0°C.

The residual activity was determined after diluting each sample to the same over-all dilution. The results showed that at 37°C, C1r activity was more stable at higher concentrations and that below 32 $C\bar{1}$ SFU/ml activity was lost progressively as the concentration decreased. No changes were found in the samples that were held at 0°C.

Purification of C1r. Euglobulin was precipitated overnight from 500 ml of fresh normal human serum or 1000 ml of serum from recalcified, clotted, outdated blood[5] by the method described by Tamura and Nelson (17) at 0.04 RSC, pH 7.5, and 0.005 M phosphate buffer. The precipitate was centrifuged for 1 hr at 1370 *g* in a PR2 (International Equip., Needham Heights, Mass.) centrifuge and washed two times with 0.04 RSC, 0.005 M phosphate buffer pH 7.5. Finally it was suspended in the starting buffer for the DEAE column: 0.110 RSC, 0.005 M Tris-HCl buffer, and pH 8.3 containing 0.01 M EDTA. The neutral euglobulin sample, concentrated 5 times with respect to the original serum volume, was applied to a 5.5 × 30 cm column of DEAE-cellulose (DE-23 Whatman) equilibrated with starting buffer, and the column was washed with 3 volumes of the same buffer. A NaCl gradient prepared with 2 liters of 0.25 M NaCl in starting buffer and 1.0 liter of starting buffer was begun. C1r activity was located in the eluted fractions using a rapid scanning method of assay: one drop (0.021–0.025 ml) of a 1/100–1/1000 dilution of the fractions in $DGVBS^{++}$ was added to one drop of C1q, 0.40 μg/ml, and one drop of C1s, 0.1 μg/ml. The C1q, C1r, and C1s mixture was incubated at 30°C for 10 min, cooled at 0°C, and 0.1 ml of EAC4 at a concentration of 1.5×10^8 cells/ml was added. After 10 min of incubation at 30°C the cells were washed once with $DGVBS^{++}$ and resuspended in 0.5 ml $DGVBS^{++}$. One drop of C2 containing 50 SFU/ml was added, and after 10 min of incubation at 30°C, 2.0 ml of 1/100 C-EDTA was added and the reaction tubes were transferred to the 37°C bath for approximately 15 min. The rate and extent of lysis are proportional to the amount of C1r. As shown in Fig. 1, the major portion of the C1r activity eluted from 0.150 to 0.180 RSC.

DEAE column fractions containing C1r eluting before the peak of C1s activity were pooled and the pH adjusted to pH 5.0 with 0.2 M acetic acid. (Note that some C1s always eluted with C1r.) The RSC of the pool was adjusted to 0.03 by dilution at 0°C and the diluted sample was applied to a 2.5 × 30 cm CM-cellulose column (CM-23 Whatman) equilibrated with 0.030 RSC, pH 5.0, and 0.02 M acetate buffer. The column was washed with 2 volumes of this buffer and a gradient made with 1 liter of 0.10 M NaCl in starting buffer and

[5] The yield of C1r activity was lower when outdated blood was used.

0.5 liter of starting buffer was begun, collecting 7 ml fractions. Peak C1r activity eluted at a RSC of 0.085, starting at 0.06 RSC. (Fig. 1) With these conditions the main peak of C1s eluted at 0.03 RSC. The fractions were scanned for C1r activity as described above. Fractions containing C1r activity were carefully pooled to avoid including those that were contaminated with the protein peak that elutes at 0.095 RSC.

The OD_{280} of this post CM C1r pool was below 0.005, and the number of $C\bar{1}$ SFU/ml of C1r ranged in various runs from 20,000 $C\bar{1}$ SFU/ml when fresh serum was used as starting material to 400 $C\bar{1}$ SFU/ml when outdated blood was used. The C1r pool was concentrated and dialyzed against VBS and stored at −70°C.

Further purification of C1r was obtained by preparative disc acrylamide electrophoresis. In this way the contaminating albumin and traces of residual C1s were removed. This is necessary before using these preparations for immunization of rabbits. Antisera have been produced in rabbits and will be described later. Acrylamide gel electrophoresis was carried out for 4 hr at 175 v or 15 hr at 100 v. Gels were sliced in 2 mm segments, eluted with 0.15 M NaCl and the C1r activity was determined after 2–24 hr elution. A correlation of activity with protein stain was obtained by slicing the gels longitudinally before elution. The recovery after 3 days of elution was 30–58% of the total activity applied. As shown in Fig. 1, the activity corresponded to the relatively diffuse protein band in the slow β-globulin zone. It was necessary to maintain the temperature at 0°–4°C during the electrophoretic run to prevent inactivation of C1r.

The estimated recovery after each chromatographic step was 20–40%. Loss of activity was most likely to occur during steps when C1r was very dilute.

Analytical disc acrylamide electrophoresis. The electrophoretic position of C1r on polyacrylamide gels was surveyed at different stages of purification. As shown in Figs. 2 *b* and 2 *c*, $C\bar{1}r$ post-DEAE and C1r post-CM were analyzed on acrylamide gels (0.5 × 4 cm running gel phase, 0.5 × 1 cm stacking gel phase) at 175 v for 2 hr at 4°C. The gels were sliced longitudinally and half was stained. The other portion was sliced in 2 mm segments and eluted with 0.5 ml of NaCl 0.15 M for 2 hr. The position of the eluted C1r activity was related to the electrophoretic pattern and to the mobility of C1s. Thus C1r activity was found at each stage of purification in a position slightly anodal to the IgG zone, and its R_f in relation to C1s was 0.294. As can be seen in Fig. 2 *c*, when purified post-CM fractions are subjected to electrophoresis the C1r activity correlates with a broad protein band which has a diffuse edge towards the anode. If the gel segments containing C1r activity were positioned on the top of a second gel and a second electrophoresis was carried out, C1r activity was again

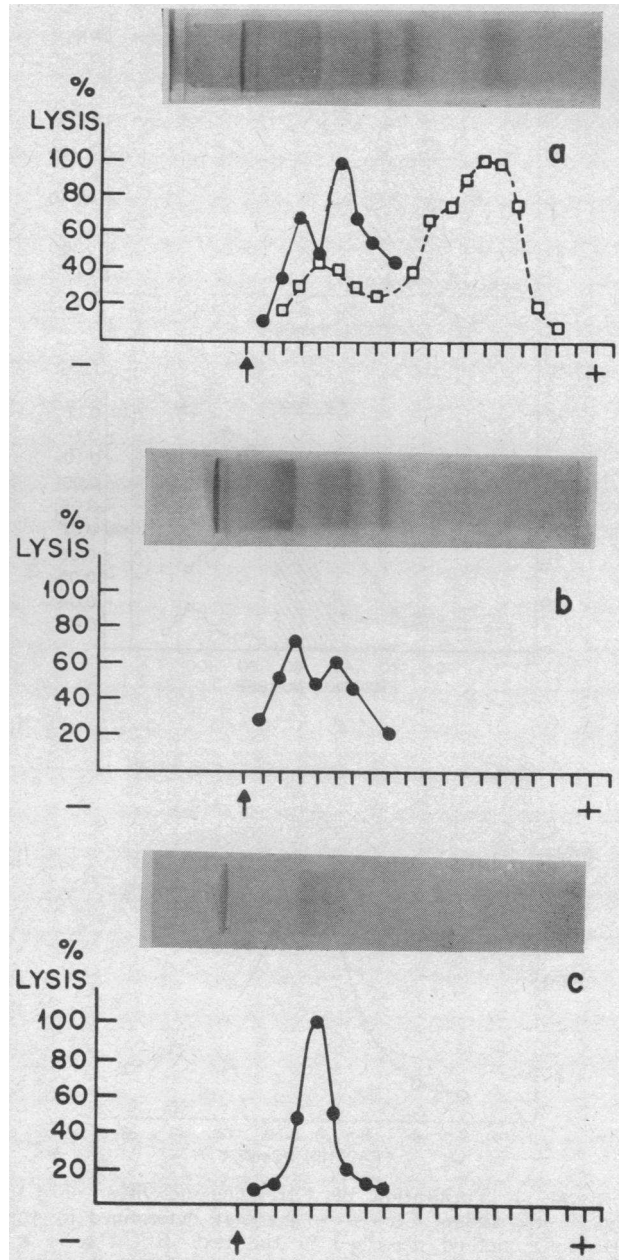

FIGURE 2 Analytical disc acrylamide electrophoresis. Protein patterns and the location of C1r activity ●——● eluted from disc acrylamide gels at various steps of purification are shown: (*a*) neutral euglobulin, (*b*) post-DEAE, (*c*) post-CM cellulose. Activity of C1s, □——□ was determined in the eluates from (*a*). The stained portion of each gel appears above its activity pattern. Origins of gels are indicated by the arrow.

associated with this protein band. When less purified preparations were applied on the gels (euglobulin and post-DEAE samples, Figs. 2 *a* and *b*), it was repeatedly found that the elution profile of C1r activity showed a depression in the center of the peak. However, when

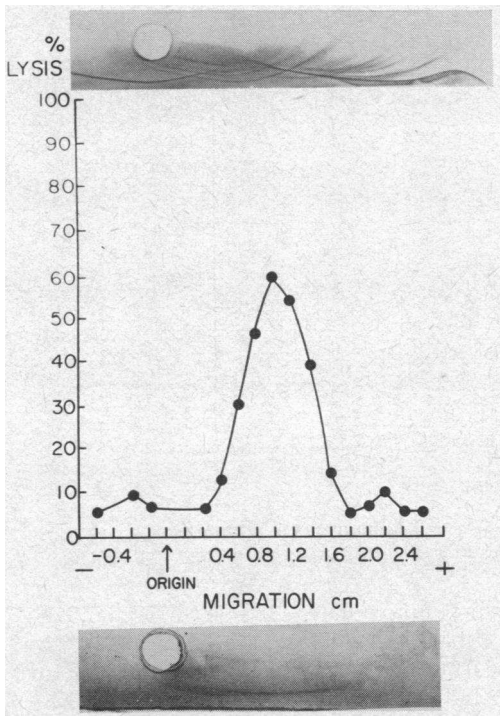

FIGURE 3 Mobility of C1r in 1% agarose, pH 8.6, EDTA 0.01 M 4°C. Crude C1r was applied to each of the five central wells of a 7.5 × 10 × 0.2 cm agarose block. Whole human serum containing 0.01 M EDTA was placed in the two outside wells. After 2 hr at 3 v/cm the central portion was sliced in 0.2 cm segments and eluted. Horse anti-human serum was placed in the upper trough and rabbit antiserum produced by immunization with acrylamide segments containing C1r in the lower trough.

the C1r-containing segments were electrophoresed for a second time the activity eluted in a single symmetric peak whose center corresponded with that of highly purified C1r. This slight depression may represent trace amounts of the C1s inhibitor (24) in the less purified samples. Because Ratnoff, Pensky, Ogston, and Naff have shown that C1s inhibitor inhibits the enzymatic activity of C1r (25), C1s inhibitor might be expected to decrease the hemolytic activity of C1r.

Mobility of C1r in agarose electrophoresis. In order to determine the electrophoretic mobility of C1r in relation to other serum proteins, a 7.5 × 0.2 × 10 cm 1% agarose block was poured on a glass slide. The buffer was 0.05 M Veronal at pH 8.6 containing 0.01 M EDTA. Seven 4 mm wells were cut and the center five wells were filled with crude C1r. The two outside wells contained whole human serum to which EDTA to a final concentration of 0.01 M was added. After electrophoresis for 2 hr at 3 v/cm at 4°C, the center portion was sliced into 2 mm segments. Each segment was eluted with 0.5 ml of 0.15 M NaCl and the C1r activity determined after 2 hr of elution at 4°C. As shown in Fig. 3, the activity of C1r migrates as a β-globulin. The relative mobility of C1r in relation to albumin was 0.4 at these conditions. The upper and lower portions of the agarose gel containing human serum were developed for 24 hr using the forced diffusion method of van Oss and Bronson (22) to increase the sensitivity. Horse antiserum against human serum was placed in the upper trough, and an antiserum prepared by immunizing the rabbits with highly purified C1r was placed in the lower trough. In the latter case, only one line corresponding with the zone of C1r activity could be demonstrated after 24 hr of diffusion as shown in Fig. 3. Characterization of this antibody is now in progress. A post-CM preparation of C1r containing 13,300 $C\bar{1}$ SFU/ml and 0.120 OD_{280} U/ml did not react with horse anti-human serum. However, it showed one band migrating to the same position as the band seen with human serum when rabbit antiserum against highly purified C1r was placed in the trough. Ouchterlony analysis of this C1r preparation concentrated 10-fold (1.2 OD_{280} U/ml) yielded three bands against anti-human serum. Faint reactions were observed with anti-C1s and anti-C3, and a strong reaction was obtained with anti-human serum albumin. These proteins represent trace contaminants at that stage of purification and emphasize the need for the final acrylamide step to prepare C1r for use as an antigen.

Sephadex G-200 gel filtration. In order to estimate the molecular weight of C1r, 0.25 ml of crude C1r was applied at 4°C to a 1 × 50 cm Sephadex G-200 column equilibrated with VBS without gelatin or metals. 0.65 ml fractions were collected and assayed for C1r activity. The column was calibrated with ^{125}I-labeled human serum albumin and cytochrome *c*.

Since crude C1r is contaminated with IgG, the elution position of IgG was assayed with a sensitive radial immunodiffusion method and the value was used as an additional protein to calibrate the column. The results are shown in Fig. 4. Using the method of Andrews (26), the calculated molecular weight of C1r was 168,100.

C1r assay. The formation of macromolecular $C\bar{1}$ in the fluid phase in the presence of excess amounts of C1q and C1s forms the basis for this hemolytic assay of C1r and in this regard is similar to the method described by Lepow et al. (7).

TABLE III
C1r Assay Method

Step	Temperature and time
1. C1q + C1r + C1s → $C\bar{1}$	30°C, 45 min
2. $C\bar{1}$ + EAC_4 → $EAC\bar{1}4$	30°C, 10 min
3. Wash (2x) $EAC\bar{1}4$	Room temperature
4. $EAC\bar{1}4$ + C2 → $EAC\overline{1\bar{4}2}$	30°C, 10 min
5. $EAC\overline{1\bar{4}2}$ + C3-9 → Hemolysis	37°C, 90 min

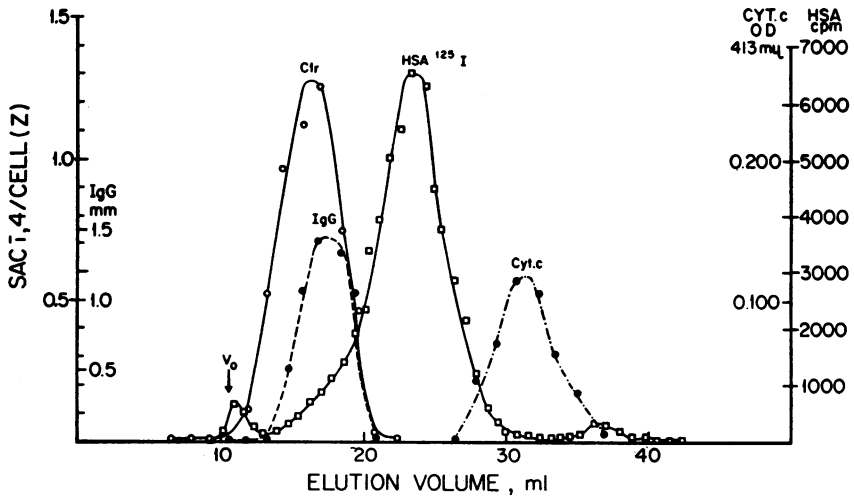

FIGURE 4 Sephadex G-200 gel filtration of crude C1r. ^{125}I human serum albumin cytochrome *c* and human IgG were used as markers as described in the text.

The C1r assay method is outlined in Table III. The buffer was DGVBS^{++}. Fully activated $C\bar{1}s$ was used. After the mixture of C1q, C1r, and $C\bar{1}s$ was incubated at 30°C for 45 min (see below), it was diluted. 0.25 ml of the dilution was then added to an equal volume of EAC4 cells at a concentration of 1.5×10^8/ml for 10 min at 30°C. The resultant $EAC\bar{1}4$ were washed twice at room temperature to remove excess $C\bar{1}s$ which would form $SAC\overline{42}$ sites, leading to an overestimation of $C\bar{1}$ molecules. After washing, the $EAC\bar{1}4$ were suspended in 0.5 ml of DGVBS^{++} and converted to $EAC\overline{1\bar{4}2}$ by incubation at 30°C for 10 min with 0.25 ml of a dilution of C2 containing 50 SFU/ml. Then 3.0 ml of C-EDTA diluted 1/50 were added and the effective number of $C\bar{1}$ molecules calculated from the per cent of cells hemolyzed (13). The C1r samples were always diluted initially so that a relative excess of C1q and $C\bar{1}s$ would be present. The maximal $C\bar{1}s$ concentration was fixed by the necessity of eliminating residual unreacted $C\bar{1}s$ with two buffer washes. More extensive washing would make the assay unduly cumbersome. $C\bar{1}s$, nonspecifically adsorbed to the reaction tube, is capable of producing $SAC\overline{42}$ sites. The $C\bar{1}s$ reagent did not contain C1q or C1r, and although the C1q reagent had traces of C1r and $C\bar{1}s$, it met the criteria for functional purity described by Nelson et al. (19). Therefore the total concentration was kept sufficiently low in order that the $C\bar{1}s$, C1q control tube would have fewer than 0.005 $C\bar{1}$ sites/cell after the two washes (i.e. less than 5% lysis). The following experiments were done to determine the optimal time for incubating C1q, C1r and $C\bar{1}s$ and the shape of the dose-response curve.

Optimal time of incubation. A mixture of 0.1 ml C1q (19.8 μg/ml), 0.1 ml $C\bar{1}s$ (0.8 μg/ml), and 0.1 ml of C1r containing approximately 450 $C\bar{1}$ SFU/ml in DGVBS^{++} was incubated at 30°C. At 10, 30, 60, 120, and 180 min a 0.010 ml sample was added immediately to 1.5 ml of 0°C DGVBS^{++}. 0.25 ml of this dilution was added to 0.25 ml of EAC4, 1.5×10^8/ml, and incubated for 10 min at 30°C. The resultant $EAC\bar{1}4$ cells were washed two times with DGVBS^{++} and then C2 and C-EDTA were added stepwise to determine the number of $C\bar{1}$ sites (13).

The results in Fig. 5 show that the optimal time of incubation is between 30 and 60 min. A longer incubation time results in a slight loss of activity possibly due to instability of $C\bar{1}$ or one of its subunits.

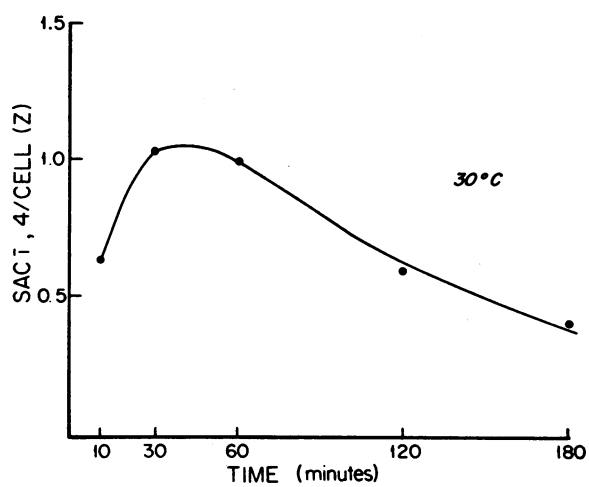

FIGURE 5 Kinetics of $C\bar{1}$ formation. A mixture of C1q (19.8 μg/ml) and C1s (0.8 μg/ml) was incubated at 30°C with purified C1r. At various times the mixtures were diluted and mixed with EAC4 for 10 min at 30°C. The number of $SAC\bar{1}4$ was determined after two washes (see text).

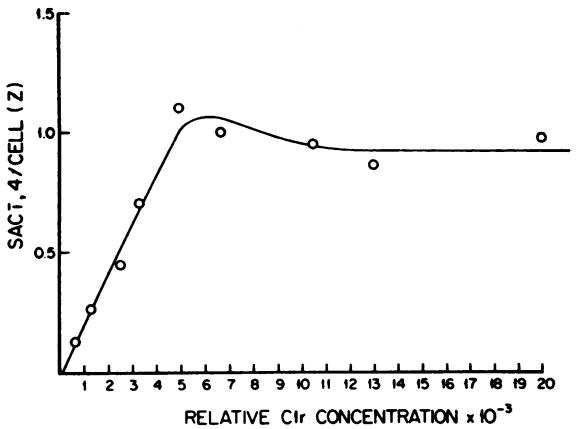

FIGURE 6 Effective $C\bar{1}$ molecules formed at various C1r concentrations. Equal volumes of C1q (9.9 μg/ml) and $C\bar{1}s$ (0.4 μg/ml) were incubated with several dilutions of C1r for 45 min at 30°C. After incubation the effective number of $C\bar{1}$ molecules formed in the incubating mixture was determined using the assay described in the text.

C1r: dose response. By adding varying amounts of C1r to constant amounts of C1q and $C\bar{1}s$, the dose-response curve shown in Fig. 6 was determined. In this experiment equal volumes, containing 9.9 μg/ml of C1q and 0.4 μg/ml of $C\bar{1}s$ and various concentrations of C1r were mixed and incubated for 45 min at 30°C. The reaction mixtures were diluted 600-fold and the newly formed $C\bar{1}$ molecules were assayed in the same manner described above. The dose-response curve is linear up to 636 $C\bar{1}$ SFU/ml of C1r at which level it tends to plateau, suggesting a limiting concentration of C1q or $C\bar{1}s$ at this point. As the number of C1q or $C\bar{1}s$ molecules in the incubation mixture was more than the number of newly formed $C\bar{1}$ molecules, an inefficiency of combination with C1r or a multiplicity of C1q or C1s subunits in each $C\bar{1}$ molecule is suggested. Direct assay shows that the number of $C\bar{1}s$ is less than two (27) in an $EAC\bar{1}4$ site. Alternatively, because C1q is relatively labile antigenically, as noted by Hanauer and Christian (1), and functionally our first thought was that some of the C1q is inactive. Specific activities of C1q preparations require further study. Specific activities of $C\bar{1}s$ have been determined and are relatively constant (12).

The dose response of $C\bar{1}$ formed from purified subunits was determined in another experiment. Our interest was stimulated by the fact that C1 in whole serum does not give a linear dose-response curve using the assay method described by Borsos and Rapp (13). On the other hand most of the partially purified C1 preparations give a linear-titration curve, but some require activation. Deviations from linearity have been tentatively interpreted as evidence of a reversible association of subunits and/or activation of at least one from a precursor state (28).

The dose-response behavior of the $C\bar{1}$ formed by combination of the three highly purified subunits at different subplateau concentrations of C1r was determined (Fig. 7). The C1q and $C\bar{1}s$ concentrations were the same as used in the preceding experiment. Since

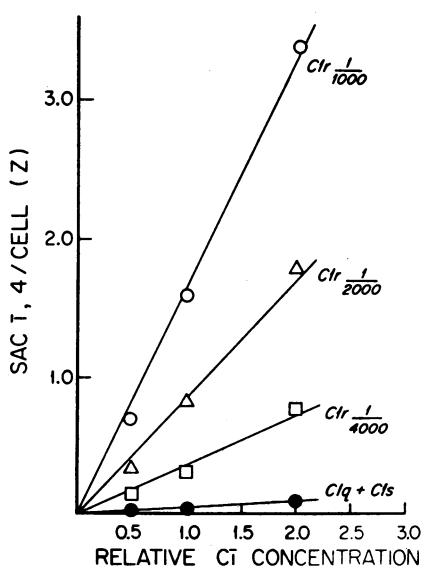

FIGURE 7 $C\bar{1}$ dose response profile using $C\bar{1}$ formed with purified C1r (constant C1q and C1s). Equal volumes of C1q (9.9 μg/ml), $C\bar{1}s$ (0.4 μg/ml), and C1r dilutions, 1/1000, 1/2000, or 1/4000 were incubated at 30°C for 1 hr. Various dilutions of these incubation mixtures were then added to EAC4 (1.5×10^8/ml) and after 10 min at 30°C the $EAC\bar{1}4$ were washed twice and the $SAC\bar{1}4$ were assayed. The lower curve refers to the C1q, C1s buffer control (see text).

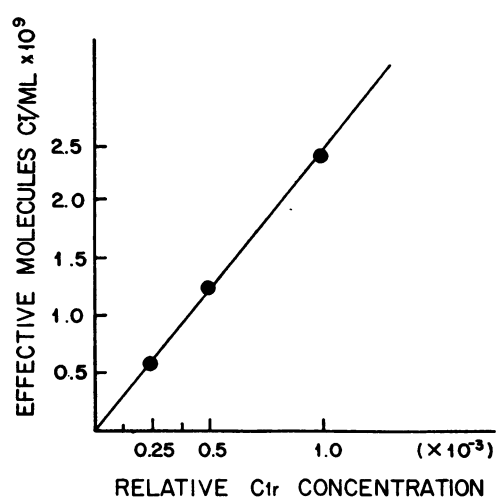

FIGURE 8 Effective $C\bar{1}$ molecules formed at various C1r concentrations. Using the data in Fig. 7 the number of effective $C\bar{1}$ molecules determined by the method of Borsos and Rapp (13), is plotted on the ordinate as a function of the C1r concentration in the mixture with C1q and $C\bar{1}s$.

C1r was more dilute in this experiment fewer C1 molecules were generated and thus large dilutions of the C1q, C1r, and $C\bar{1}s$ reaction mixture were not possible. Therefore the control tube consisting of C1q, $C\bar{1}s$, and buffer reflects this increase and 0.15 $SAC\bar{1}4$/cell were found instead of the usual 0.005 $SAC\bar{1}4$/cell' as can be seen in Fig. 7. This represents free C1s adsorbed to the reaction tube, as discussed above, and perhaps a weak C1q, $C\bar{1}s$ interaction. Note that the $C\bar{1}$ generated by combination of the three subunits shows a linear-titration curve. If the recombined subunits underwent a reversible interaction we would not expect a linear response.

As expected, the number of $C\bar{1}$ macromolecules obtained in this last experiment was linearly related to the concentration of C1r (Fig. 8). This relationship has always been found provided C1r is diluted below the plateau point. The assay method as outlined, in Table III is useful for purification and mechanism studies as it is reproducible and the response is stoichiometrically related to the C1r concentration.

DISCUSSION

C1r is one of the last complement components to be obtained in a highly purified state. Its purification was handicapped by its instability, low serum concentration, and physicochemical properties similar to many other proteins in human serum. The stability data that we have presented indicate that careful selection of pH, ionic strength, and temperature allow a high degree of purification while preserving the hemolytic activity. The neutral euglobulin preparation described by Tamura and Nelson is a suitable starting material for chromatography since it contains 90–99% of the C1 activity and less than 1% of the serum protein (17). 99% of the other eight complement components remain in the supernatant phase, although traces of C4, C5, and C3 are known to be contaminants of this euglobulin precipitate (17).

As C1r is a β-globulin and most of the complement components as well as many unrelated human serum proteins are also β-globulins, this first purification step is important because it eliminates a major portion of the serum contaminants.

C3 and C4 can be removed by careful chromatography as suggested by Lepow et al. (7), and C1q is easily separated from C1r due to its markedly different size and charge. The last acrylamide step in the fractionation procedure is necessary to obtain a high degree of purification for immunization. Trace contaminants of C3, C1s, and albumin can be removed readily because of their faster mobility. The acrylamide segments containing C1r may be emulsified with Freund's adjuvant for immunization. Previously a similar method to obtain monospecific antibody to $C\bar{1}s$ was used (12). Characterization of the antisera produced against these C1r preparations is still in process. The antisera give one precipitation line with these highly purified preparations, one line with whole human serum and do not react with serum from patient C. C., known to be functionally deficient in C1r (15).

Using highly purified C1r, we were able to study some of its physiochemical properties. The molecular weight of C1r determined by Andrews' method is 168,100. This agrees with data of Naff, Pensky, and Lepow (29) showing that the sedimentation of C1r activity in sucrose density gradients corresponded to that of a 7S protein marker. C1r behaved as a β-globulin in agarose electrophoresis at pH 8.6 in the presence of EDTA. However, if C1q and C1s were added and the electrophoretic run was performed in the presence of Ca^{++}, no C1r activity was found in the β-globulin zone. On the contrary, $C\bar{1}$ activity was present at the application point suggesting that reformation of macromolecular C1 had taken place. A similar experiment has been done with $C\bar{1}s$ and its monospecific antisera (30). The electrophoretic mobility of C1r on acrylamide gels was that of a slow β-globulin and, characteristically, a diffuse pattern was found (Fig. 2) suggesting microheterogeneity.

The hypothesis that C1r may be an enzyme capable of activating C1s from its precursor form to its active form was suggested by Naff and Ratnoff (11). These authors demonstrated that C1r preparations were capable of hydrolyzing selected synthetic substrates. This enzymatic activity was related to the ability of C1r preparations to activate C1s. These authors could not demonstrate generation of hemolytic activity in mixtures of C1s and C1r unless C1q was also present. More study with this purified C1r preparation should enable direct study of the conversion of C1s and isolation of the conversion products.

Gigli and Austen (31) have proposed, based on their studies of the reaction of $C\bar{1}s$ with C2 in the fluid phase, that C1r regulates the enzymatic activity of $C\bar{1}s$ allosterically obscuring the affiinity of $C\bar{1}s$ for C2. C1q is not necessary for this regulation, and inactive C4 increases the specificity of $C\bar{1}s$ for C2 even in the presence of C1r. This question of whether C1r modulates the activity of $C\bar{1}s$ on C2 is important since this would influence the rate of $C\overline{42}$ convertase formation and in turn the cleavage of C3 with the resultant anaphylatoxin and chemotactic activities.

The three C1 subunits are combined as a macromolecule with a sucrose density sedimentation constant of 18S in normal human serum (29). This complex can be dissociated by the action of EDTA. Furthermore, in a patient (C.C.) with glomerulonephritis serum C1r activity was undetectable in the presence of normal C1q

and normal or low C1s (15). The $C\bar{1}$ hemolytic titer in the serum of this patient was extremely low and the activities of C1q and C1s sedimented as independent molecules even in the presence of Ca^{++}, contrary to normal human serum. These reports suggest that C1r may link C1q to C1s, although a weaker C1q, C1s interaction is not ruled out. It does not preclude independent function of one or all of these proteins. Our studies support the concept that C1r physically links these subunits.

Although these proteins are bound functionally they may not be produced by the same cells and their synthesis may not be under the same genetic control. Studies by Stroud et al. (2) demonstrate that serum C1q and C1s are independent as normal or high levels of C1s can be present in situations in which C1q is greatly diminished.

As a quantitative hemolytic assay for C1r is necessary to study these biological questions, we investigated the optimum assay conditions. The hemolytic assay described capitalizes on the fact that macromolecular $C\bar{1}$ can be assembled from C1q, C1r and $C\bar{1}$s, and that the binding of $C\bar{1}$ to EAC4 is firm at low ionic strength. Consequently all the uncombined reagents can be washed away and titration of the bound $C\bar{1}$ may be performed by the quantitative method of Borsos and Rapp (13). Since highly purified C1q and C1s were available and their protein concentration could be measured using radial immunodiffusion, the quantities of C1q and C1s reagents used in the first step (Table III) of the assay are specified in micrograms. Although the specific activity of the $C\bar{1}$s used in these assays is known, our study of the specific activity of C1q is incomplete and is hampered by its greater lability. However, the amounts of C1q and $C\bar{1}$s used are in excess as shown by the dose response data as C1r is varied (Fig. 6). The number of $C\bar{1}$ molecules formed when C1r is added at concentrations below saturation or plateau zone is a linear function of the C1r concentration. Although this suggests that there is one C1r subunit in every $C\bar{1}$ site a more direct study is needed. The concept of a $C\bar{1}$ site-forming unit, $C\bar{1}$ SFU, is useful to express the C1r titers. A $C\bar{1}$ SFU is that amount of C1r which when added to optimal amounts of C1q and C1s forms 1.5×10^8 effective molecules of $C\bar{1}$ which in the assay represents an average of one effective molecule per cell. As constructed this assay can not be used if the C1r source contains C1q and C1s as would be the case for whole serum. Using antisera and radial immunodiffusion we plan to determine the specific activity of C1r and the serum concentration as was done for C1s (12). Also it will be important to determine the absolute number of C1q and C1r subunits in a cell bound $C\bar{1}$ molecule.

The described preparation of C1r will allow us to determine the effect of C1r on C1s and the precise mechanism by which C1s is activated in diseases such as rheumatoid arthritis (synovial fluid), systemic lupus erythematosus, acute poststreptococcal glomerulonephritis and hereditary angioedema. For instance the clinical and biochemical studies of the latter condition suggest a multiple step phenomenon (6). Studies by others on activation suggest that the ionic strength and Ca^{++} concentrations are important (32). Activation can also occur with proteolytic enzymes not known to be in the complement system, such as plasmin, trypsin, lysosomal enzymes, and Hageman factor (33, 34, 10). It is interesting that Ca^{++} has been shown to have a sparing effect on $C\bar{1}$ activity. We have preliminary evidence that this effect of Ca^{++} may be on C1r. When a neutral euglobulin preparation of $C\bar{1}$ is incubated in 0.01 M EDTA at low ionic strength, at 30°C all the $C\bar{1}$ activity is lost. This activity can be restored by adding purified C1r. Furthermore, if the EDTA inactivated $C\bar{1}$ is subjected to DEAE-cellulose chromatography C1r activity cannot be detected. More precise information on the role of Ca^{++} is necessary.

ACKNOWLEDGMENTS

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REFERENCES

1. Hanauer, L. B., and C. L. Christian. 1967. Clinical studies of hemolytic complement and the 11S component. *Amer. J. Med.* **42**: 882.
2. Stroud, R. M., K. Nagaki, R. J. Pickering, H. Gewurz, R. A. Good, and M. D. Cooper. 1970. Subunits of the first complement component in immunologic deficiency syndromes: Independence of C1s and C1q. *Clin. Exp. Immunol.* **7**: 133.
3. Kohler, P. F., and H. J. Müller-Eberhard. 1967. Immunochemical quantitation of the third, fourth and fifth components of human complement: Concentrations in the serum of healthy adults. *J. Immunol.* **99**: 1211.
4. Becker, E. L. 1956. Concerning the mechanism of complement action. I. Inhibition of complement activity by Diisopropyl fluorophosphate. *J. Immunol.* **77**: 462.
5. Bokisch, V. A., H. J. Müller-Eberhard, and C. G. Cochrane. 1969. Isolation of a fragment (C3a) of the third component of human complement containing anaphylatoxin and chemotactic activity and description of an anaphylatoxin inactivator of human serum. *J. Exp. Med.* **129**: 1109.
6. Donaldson, V. H., O. D. Ratnoff, W. D. DaSilva, and F. S. Rosen. 1969. Permeability increasing activity in hereditary angioneurotic edema plasma. II. Mechanism of formation and partial characterization. *J. Clin. Invest.* **48**: 642.
7. Lepow, I. H., G. B. Naff, E. W. Todd, J. Pensky, and C. F. Hinz, Jr. 1963. Chromatographic resolution of the first component of human complement in three activities. *J. Exp. Med.* **117**: 983.
8. Lepow, I. H., L. Pillemer, and O. D. Ratnoff. 1953. The influence of calcium ions on the inactivation of human

complement and its components by plasmin. *J. Exp. Med.* **98:** 277.
9. Lepow, I. H., L. Wurz, O. D. Ratnoff, and L. Pillemer. 1954. Studies on the mechanism of inactivation of human complement by plasmin and by antigen-antibody aggregates. *J. Immunol.* **73:** 146.
10. Donaldson, V. H. 1968. Mechanism of activation of C'1 esterase in hereditary angioneurotic edema plasma *in vitro*: Role of Hageman factor, clot promoting agent. *J. Exp. Med.* **127:** 411.
11. Naff, G. B., and O. D. Ratnoff. 1968. The enzymatic nature of C1r. Conversion of C1s and C1 esterase and digestion of amino acid esters by C1r. *J. Exp. Med.* **128:** 571.
12. Nagaki, K., and R. M. Stroud. 1970. A method for preparing monospecific antisera to C1 esterase (C1s): Microheterogeneity of purified C1s. *J. Immunol.* **105:** 162.
13. Borsos, T., and H. J. Rapp. 1963. Chromatographic separation of the first component of complement and its assay on a molecular basis. *J. Immunol.* **91:** 851.
14. Nagaki, K., and R. M. Stroud. 1969. The relationship of the hemolytic activity of active C1s to its TAMe esterase action: A new method of purification and assay. *J. Immunol.* **102:** 421.
15. Pickering, R. J., G. B. Naff, R. M. Stroud, R. A. Good, and H. Gewurz. 1970. Deficiency of C1r in human serum. Effects on the structure and function of macromolecular C1. *J. Exp. Med.* **131:** 803.
16. Mayer, M. M. 1961. *In* Kabat and Mayer's Experimental Immunochemistry. 2nd Edition. Charles Thomas, Springfield, Illinois, 133.
17. Tamura, N., and R. A. Nelson, Jr. 1968. The purification and reactivity of the first component of complement from guinea pig, human and canine sera. *J. Immunol.* **101:** 1333.
18. Müller-Eberhard, H. J. 1968. Chemistry and reaction mechanisms of complement. *Advan. Immunol.* **8:** 1.
19. Nelson, R. A., Jr., J. Jensen, I. Gigli, and N. Tamura. 1966. Methods for the separation, purification and measurement of nine components of hemolytic complement in guinea-pig serum. *Immunochemistry.* **3:** 111.
20. Borsos, T., H. J. Rapp, and M. M. Mayer. 1961. Studies on the second component of complement. I. The reaction between EAC1,4 and C2: Evidence on the single site mechanism of immune hemolysis and determination of C2 on a molecular basis. *J. Immunol.* **87:** 310.
21. Mancini, G., A. O. Carbonara, and J. F. Heremans. 1965. Immunochemical quantitations of antigens by single radial immunodiffusion. *Immunochemistry.* **2:** 235.
22. van Oss, C. J., and P. M. Bronson. 1969. Immunorheophoresis. *Immunochemistry.* **6:** 775.
23. Davis, B. J. 1964. Disc electrophoresis. II. Method and application to human serum protein. *Ann. N. Y. Acad. Sci.* **121:** 404.
24. Pensky, J., L. R. Levy, and I. H. Lepow. 1961. Partial purification of a serum inhibitor of C1 esterase. *J. Biol. Chem.* **236:** 1674.
25. Ratnoff, O. D., J. Pensky, D. Ogston, and G. B. Naff. 1969. The inhibition of plasmin plasma kalikrein, plasma permeability factor and the C1r subcomponent of the first component of complement by serum C1 esterase inhibitor. *J. Exp. Med.* **129:** 315.
26. Andrews, P. 1964. Estimation of the molecular weight of proteins by Sephadex gel filtration. *Biochem. J.* **91:** 222.
27. Stroud, R. M., M. Bracco, and K. Yonemasu. 1970. Radioimmunoassay for C1 esterase (C1s). *Fed. Proc.* **29:** 330 (Abstr.)
28. Hoffmann, L. G. 1970. Reaction of native guinea pig C1 with sensitized erythrocytes. *Fed. Proc.* **29:** 303 (Abstr.)
29. Naff, G. B., J. Pensky, and I. H. Lepow. 1964. The macromolecular nature of the first component of human complement. *J. Exp. Med.* **119:** 593.
30. Nagaki, K., and R. M. Stroud. 1969. Specific antisera to C1s: Detection of different electrophoretic species of C1s. *J. Immunol.* **103:** 141.
31. Gigli, I., and K. F. Austen. 1969. Fluid phase destruction of $C2^{hu}$ by $C1^{hu}$. II. Unmasking by $C4i^{hu}$ of $C1^{hu}$ specificity for $C2^{hu}$. *J. Exp. Med.* **130:** 833.
32. Ratnoff, O. D., and G. B. Naff. 1969. The role of C1q, cations, and ionic strength in the formation of C1 esterase. *J. Lab. Clin. Med.* **74:** 380.
33. Ratnoff, O. D., and G. B. Naff. 1967. The conversion of C1s to C1 esterase by plasmin and trypsin. *J. Exp. Med.* **125:** 337.
34. Taubman, S. B., P. R. Goldschmidt, and I. H. Lepow. 1970. Effects of lysosomal enzymes from human leukocytes on human complement components. *Fed. Proc.* **29:** 434 (Abstr.)