The Third Component of the Guinea Pig Complement System. I. Purification and Characterization*

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ABSTRACT: C'3 has been isolated from the pseudoglobulin fraction of guinea pig serum by DEAE- and CM-cellulose chromatography, and by Pevikon block electrophoresis.

The final product contains no impurity detectable by any of these fractionation procedures or by hydroxylapatite chromatography, immunoelectrophoresis, or disc electrophoresis. Purified C'3 has a sedimentation coefficient of 7.4 S and its molecular weight is 180,000 by sedimentation equilibrium analysis. The final product does not contain any of the other eight components of the complement system.

The complement system of guinea pig blood serum comprises nine factors which are designated C'1,[1] C'4, C'2, C'3, C'5, C'6, C'7, C'8, and C'9, according to their sequence of action in hemolyzing erythrocytes sensitized with antibody. While there is much information on C'1, C'4, and C'2, the properties and reactions of the other factors are still poorly understood. Accordingly, we have studied C'3 and as a first step, we wish to present a procedure for the isolation of this factor in pure form, as judged by chemical and biological criteria. While methods have been described for the preparation of functionally pure guinea pig C'3 (Nelson *et al.*, 1966) and chemically homogeneous human C'3 (Nilsson and Müller-Eberhard, 1965), a procedure for the isolation of functionally and chemically pure guinea pig C'3 has not been described heretofore.

Methods

The hemolytic activity of C'3 was measured by the method described in the accompanying paper (Shin and Mayer, 1968b).

Purification of C'3. Pooled guinea pig serum was purchased from the Baltimore Biological Laboratories, Baltimore, Md., and stored at −40°. DEAE-cellulose (Brown Paper Co., Berlin, N. H., lot 1531, 0.88 mequiv/g) and CM-cellulose (Brown Paper Co., lot 1464, 0.71 mequiv/g) were washed and packed into columns under gravity as described by Nelson *et al.* (1966). Pevikon particles (Stockholm Superfosfat Fabriks A-B, Stockholm, Sweden) were used for preparative electrophoresis as described by Müller-Eberhard (1960). Protein solutions were concentrated and dialyzed in the ultrafiltration apparatus supplied by Carl Schleicher & Schuell Co., Keene, N. H. All procedures were performed at 3° unless specified otherwise.

Guinea pig serum (150–200 ml) was adjusted to pH 7.5 with 1 M acetic acid and diluted with ice-cold distilled water until the conductivity corresponded to that of 0.04 M NaCl. The resulting precipitate was allowed to aggregate for 30 min and then removed by centrifugation at 1500*g*, 0°, for 45 min. The clear supernatant was applied at a flow rate of 250 ml/hr to a DEAE-cellulose column, 5.0-cm diameter and 70 cm height, equilibrated with pH 7.5 starting buffer containing 0.005 M phosphate, 0.033 M NaCl, and 0.001 M EDTA. The column was then washed with 1500 ml of starting buffer. Following this, a linear gradient was developed by gradual addition (150 ml/hr) of 1500 ml of a pH 7.5 buffer containing 0.005 M phosphate, 0.243 M NaCl, and 0.001 M EDTA to a mixing chamber containing 1500 ml of the starting buffer.

About 400 ml of eluate containing C'3 was pooled, concentrated to 10 ml by ultrafiltration, and diluted to 20 ml with an appropriate NaCl solution to adjust the conductivity to that of 0.08 M NaCl. The pH was brought to 5.0 with 1 M acetic acid. No precipitate appeared. The material was applied at a rate of 20 ml/hr to a CM-cellulose column, 2.5-cm diameter and 40 cm height, equilibrated with pH 5 starting buffer containing 0.02 M acetate and 0.07 M NaCl. The column was washed with 150 ml of starting buffer. A linear gradient was then developed by gradual addition (75 ml/hr) of 450 ml of pH 5 buffer containing 0.02 M acetate and 0.19 M NaCl to a mixing chamber containing 450 ml of starting buffer. Fractions from CM-cellulose columns were collected in tubes containing an appropriate amount of 0.5 M Tris to bring the pH to 7.5. A volumetric collecting device was used since its rapid discharge of each fraction promotes mixing with the Tris buffer.

Approximately 400–500 ml of eluate containing C'3 was concentrated to 1 ml by ultrafiltration and dialyzed

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[1] Abbreviations used that are not listed in *Biochemistry 5,* 1445 (1966), are: C', complement; A, antibody to the boiled stromata of sheep erythrocytes; S, site on the erythrocyte surface capable of reacting with A and C' components; C'*j*, *j*th component of C'; C'*j*a, activated form of C'*j*; SAC'*i*, *j*, *k*, S which has reacted with A and C'*i*, C'*j*, and C'*k*.

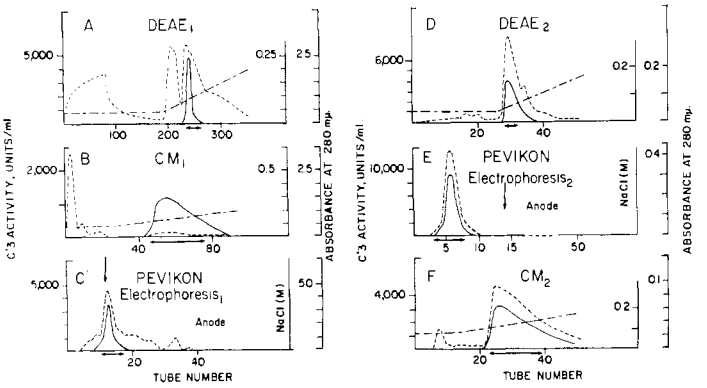

FIGURE 1: Purification steps in the isolation of C'3. Sequence of purification in alphabetical order. (A) DEAE column chromatography, (B) CM column chromatography, (C) Pevikon electrophoresis, (D) DEAE column chromatography, (E) Pevikon electrophoresis, and (F) CM column chromatography. Solid lines represent C'3 activity (units per milliliter); dots and dashes represent NaCl concentration; dotted line represents absorbance at 280 mμ. Double-headed horizontal arrows indicate tubes which were pooled for next step. Vertical arrow indicates point of sample application on Pevikon block. In four other purification runs, during the first and second Pevikon block electrophoresis, C'3 moved one to five tubes from the point of sample application toward the anode.

for 4 hr against 500 ml of Tris buffer (pH 8.6) and ionic strength 0.05. The material was applied, 12 cm from the cathode, to a Pevikon block (1 × 10 × 50 cm) equilibrated with the pH 8.6 Tris buffer. The electrophoresis was run for 48 hr at 400 V, with a current of 20 mA and a gradient of 2.5 V/cm of Pevikon block. After the electrophoresis, the block was sliced into 1 × 1 × 10 cm segments, and each segment was eluted four times in 10 ml of pH 7.5 buffer containing 0.005 M phosphate, 0.15 M NaCl, and 0.001 M EDTA. Only the first eluate of each fraction was analyzed for C'3 activity but all four eluates were pooled if the first eluate contained C'3.

For further purification, the three fractionation procedures just described were repeated, but in a different order.

A pool of about 300–500 ml of the electrophoretically fractionated C'3 was concentrated to 30 ml and diluted with 0.005 M phosphate buffer (pH 7.5) until the conductivity corresponded to that of 0.035 M NaCl. The pH was adjusted to 7.5 if necessary. The material was applied at a rate of 15 ml/hr to a DEAE column (2 × 30 cm) equilibrated with pH 7.5, starting buffer, containing 0.005 M phosphate, 0.028 M NaCl, and 0.001 M EDTA. The column was washed with 100 ml of this starting buffer and then a linear gradient was developed by the addition (30 ml/hr) of 250 ml of pH 7.5 buffer, containing 0.005 M phosphate, 0.193 M NaCl, and 0.001 M EDTA to a mixing chamber containing 250 ml of starting buffer.

Eluate (40–50 ml) containing C'3 was concentrated to 0.2 ml, followed by dialysis and electrophoresis on a 1 × 5 × 50 cm Pevikon block, as before, except that the current was 10 mA and segments of 1 × 1 × 5 cm were eluted in 5-ml portions of pH 7.5 buffer.

About 100 ml of eluate containing C'3 was concentrated to about 0.5 ml and dialyzed for 12 hr against 500 ml of pH 7.5 buffer, containing 0.005 M phosphate and 0.035 M NaCl. After dialysis, the contents of the dialysis bag were diluted with 6 ml of pH 5.0 starting buffer, containing 0.02 M acetate and 0.07 M NaCl. The material was applied to a CM-cellulose column (1 × 20 cm) equilibrated with the pH 5.0 starting buffer. The column was washed with 50 ml of the starting buffer and a linear gradient was applied by gradual addition (12 ml/hr) of 120 ml of pH 5.0 buffer containing 0.02 M acetate and 0.24 M NaCl to a mixing chamber containing 120 ml of starting buffer.

Analysis by Immunoelectrophoresis. Electrophoresis was carried out for 2 hr at 3 V/cm according to the method of Scheidegger (1955), on a supporting medium of 1% agarose (Marine Colloid, Inc.). Precipitation lines were allowed to develop for 48 hr before pictures were taken. Approximately 20 μg of purified C'3 was applied to each well. The antiserum to purified C'3 was raised in rabbits by four weekly intramuscular inoculations, each of 1.2 mg of purified C'3 in complete Freund adjuvant. Blood was drawn 7–10 days after the last injection. Rabbit antiserum to whole guinea pig serum was kindly given to us by Dr. A. G. Osler, The Johns Hopkins School of Medicine, Baltimore, Md.

Analytical Disc Electrophoresis. Disc electrophoresis on polyacrylamide gel was performed at 3–6° according to Davis (1964), with samples of 20–50 μg of purified C'3. The solution of C'3 was mixed with an equal volume of 10% sucrose and a volume not exceeding 50 μl was layered on the large pore gel. The remaining space in the tube was filled with the Tris buffer used in the electrode compartments. The upper electrode compartment was filled carefully so as not to disturb the sample layer. Analyses were set up in pairs, and one member of the pair was used for staining with Amido Black while the other was used for sectioning followed by elution of each of the segments at 3° in buffer C^{2+} (Shin and Mayer, 1968) with gentle mechanical agitation for a period of at least 30 hr. In analyses with gel made from a concentration of 3.25% acrylamide and 0.1% *N*, *N'*-methylenebisacrylamide, electrophoresis was terminated when the tracking dye, bromophenol blue, emerged from the anodal end of the small pore gel. In the case of analyses in which the concentrations of acrylamide and *N*,*N'*-methylenebisacrylamide were doubled, electrophoresis was carried out three times longer than the time required for passage of the bromophenol blue through the small pore gel.

Analytical Column Chromatography on Hydroxyapatite. Hydroxyapatite was prepared according to Miyazawa and Thomas (1965). About 2 mg of purified C'3 in a volume of 0.5 ml was dialyzed against 150 ml of pH 6.8 starting buffer containing 0.005 M phosphate and 0.15 M NaCl. The material was applied to a hydroxylapatite column (1 × 6 cm) equilibrated with the starting buffer. The column was washed with 20 ml of phosphate buffer (0.05 M, pH 6.8) and then a linear gradient was developed by gradual addition of 75 ml of pH 6.8 buffer containing 0.4 M phosphate to 75 ml of 0.05 M phosphate buffer (pH 6.8) in a mixing chamber.

Hemolytic Analysis for C' Components to Ascertain

TABLE I: Summary of C'3 Purification (Preparation 7).

Procedures	Vol (ml)	$OD_{280\ m\mu}$	C3' Concn (units/ml)	Total C'3 (units)	C3' Yield (%)	Sp Act. Concn/ $OD_{280\ m\mu}$
Guinea pig serum	180	44.7	8,640	1,560,000	100	193
Removal of euglobulin formed at 0.04 M (pH 7.5)	1,020	6.07	1,050	1,070,000	68.9	173
First DEAE column chromatography	375	1.27	2,020	758,000	48.7	1,590
First CM column chromatography	385	0.105	1,170	450,000	28.9	11,100
First Pevikon block electrophoresis	300	0.070	1,370	410,000	26.4	19,500
Second DEAE column chromatography	50	0.193	5,880	294,000	18.8	30,400
Second Pevikon block electrophoresis	6.6	1.050	31,700	209,000	13.4	39,200
Second CM column chromatography	80	0.048	1,440	115,000	7.4	30,100

TABLE II: Summary of Purification (Preparation 9).

Procedures	Vol (ml)	$OD_{280\ m\mu}$	C'3 Concn (units/ml)	Total C'3 (units)	C'3 Yield (%)	Sp Act. Concn/ $OD_{280\ m\mu}$
Guinea pig serum	200	44.3	15,500	3,100,000	100	349
Removal of euglobulin formed at 0.04 M (pH 7.5)	626	12.6	4,180	2,241,080	84.56	332
First DEAE column chromatography	420	2.28	4,000	1,340,000	54.5	1,760
First CM column chromatography	510	0.218	1,920	979,000	31.6	8,800
First Pevikon block electrophoresis	85	0.700	10,700	907,000	29.3	15,300
Second DEAE column chromatography	40	0.425	12,000	481,000	15.5	28,300
Second Pevikon block electrophoresis	144	0.075	2,180	314,000	10.1	29,000
Second CM column chromatography	107	0.075	2,220	238,000	7.6	29,600

Functional Purity. Three preparations of C'3 were analyzed for C'1 according to Borsos and Rapp (1963), C'2 according to Borsos *et al.* (1961), C'4 according to Hoffmann *et al.* (1965), and C'5, C'6, C'7, C'8, and C'9, as well as C'3 inhibitor, according to Nelson *et al.* (1966).

Sedimentation Velocity and Sedimentation Equilibrium Analyses. Purified C'3 in pH 7.5 buffer containing 0.005 M phosphate and 0.15 M NaCl was analyzed in the Spinco Model E ultracentrifuge. For sedimentation velocity analyses, concentrations of 0.4, 0.2, 0.133, and 0.1% were used. Runs were made at 47,660 rpm at 20° in a standard single-sector cell. Sedimentation equilibrium analyses were performed according to Yphantis (1964) at C'3 concentrations of 0.015 and 0.05%. The material was centrifuged at 15,200 rpm and 20°. Pictures were taken at 18, 20, and 24 hr.

Nitrogen Determination. Two preparations of C'3 (lots 7 and 9) were analyzed for nitrogen content by the micro-Kjeldahl method (Kabat and Mayer, 1961).

Results

The results of a fairly typical purification run are outlined in Figure 1 and Tables I and II. The graphs in Figure 1 show the protein and C'3 elution patterns in each

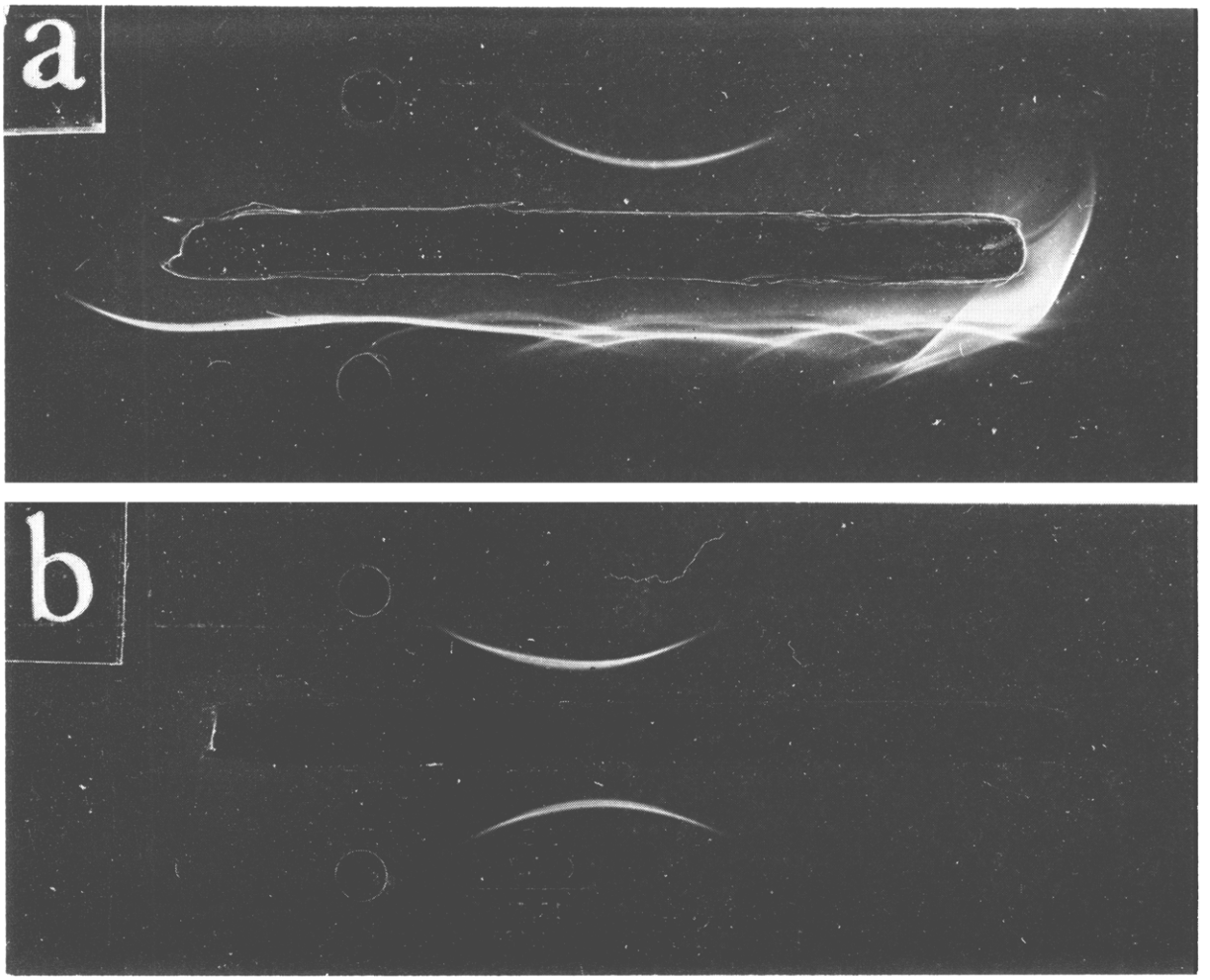

FIGURE 2: Immunoelectrophoresis of purified C′3. Slide A: upper well, purified C′3; trough, rabbit anti-guinea pig serum; lower well, guinea pig serum. Slide B: upper well, purified C′3; trough, rabbit anti-C′3; lower well, guinea pig plasma. Both buffer and samples in slide B contained 0.0005 M EDTA. Anode at right.

of the successive fractionation treatments. The fractions containing C′3 were pooled as indicated on each graph. It is evident from the elution patterns that the second zone electrophoresis and the second chromatographic fractionation on CM-cellulose did not effect further removal of substantial amounts of inactive protein. This is also evident from column 6 in Table I, as well as column 6 in Table II, which shows specific activities of C′3 at each step of the fractionation sequence. Even though no substantial quantities of inactive material were eliminated by the second zone electrophoresis and the second fractionation on CM-cellulose, these treatments were retained in the routine purification procedure in order to ensure consistent absence of impurities from the final product.

The recoveries of C′3 at each fractionation step averaged about 70%. The final yields in preparation 7 (Table I) and preparation 9 (Table II) were 7.4 and 7.6%, respectively. The specific activities of the final products were almost identical.

Test for Purity. Immunoelectrophoretic analyses of four C′3 preparations with anti-C′3 and anti-whole guinea pig serum showed single precipitation lines in every case (Figure 2). Furthermore, immunoelectrophoresis of whole guinea pig serum with anti-C′3 gave a single precipitation line (Figure 2).

Disc electrophoresis also showed a single band and C′3 activity could be eluted from the segment corresponding to this band (Figure 3). The recovery of activity was 15–20%.

Purified C′3 eluted from the hydroxylapatite column as a single symmetric peak at a phosphate concentration of 0.17 M (Figure 4). All fractions had the same specific activity. A 25% decrease of specific activity compared with starting material was noted. Recovery of activity was about 70%.

Sedimentation Velocity and Molecular Weight of C′3. The analysis by ultracentrifugation showed a single symmetric peak, as in Figure 5. The sedimentation coefficient of C′3 at 20° was 7.4×10^{-13} sec at four different concentrations, namely, 0.1, 0.125, 0.2, and 0.4%. Molecular weight values of 179,000 and 179,600 were obtained from sedimentation equilibrium analyses at concentrations of 0.05 and 0.015%, respectively. Partial specific volume of 0.725 was assumed.

Functional Purity. No other complement components were detected by hemolytic tests, as shown in Table III. C′3 inactivator was also absent.

TABLE III: Functional Purity of Three C'3 Preparations (Lots 5, 7, and 9).

Complement Factor Titrated	Titer in Purified C'3 (units)	Titer in Whole Guinea Pig Serum (units)
C'1	<1	60,000
C'4	<1	7,000
C'2	<1	10,000
C'3	3,000, 5,000, and 10,000	15,000
C'5	<1	50,000
C'6	<1	10,000
C'7	<1	40,000
C'8	<1	250,000
C'9	<1	270,000
C'3 Inactivator	<1	Not done

Absorbance at 280 mμ. When adjusted to absorbance of 1.00 at 280 mμ (1.00-cm cuvet), C'3 lots 7 and 9 contained 0.120 and 0.122 μg of N/ml, respectively. For conversion from N into protein, the factor 6.25 was used.

Discussion

C'3 can be isolated from guinea pig serum in pure form as judged by chemical and biological criteria. The final yields have ranged in the vicinity of 7% and the specific hemolytic activity of different preparations has been reasonably constant. The relative ease with which this component of the complement system can be purified is attributable, in part, to its high concentration in guinea pig serum. On the basis of the assumption that our product is indeed pure, it can be estimated that the approximate concentration of C'3 is 30–50 mg/100 ml of guinea pig serum. Furthermore, isolation of C'3 is facilitated by its relatively good stability.

Some difficulty was encountered in several of the early purification experiments in which we attempted to isolate C'3 from the euglobulin fraction precipitated from guinea pig serum at pH 5 and ionic strength 0.02. Products obtained in this manner were contaminated with an inhibitor of C'3 and it is for this reason that we changed our approach so as to start the purification with the pseudoglobulin fraction obtained at pH 7.5 and ionic strength 0.04. This change did not entail any sacrifice of yield.

The absence of impurities in our final product has been ascertained by a series of physical, chemical, and immunological analyses which were selected so as to be separate and distinct from the preparative methods and manipulations. Thus, we have obtained information on absence of impurities which is entirely independent of the evidence emerging from the preparative fractionations, as shown in Figure 1. In addition, we have established "functional purity" by biologic tests for hemolytic activity attributable to any of the other eight complement components. The absence of such "functional" contamination, as shown in Table III, is important since the sensitivity of hemolytic tests for contaminating complement components is often greater than that of physical, chemical, or immunological analyses.

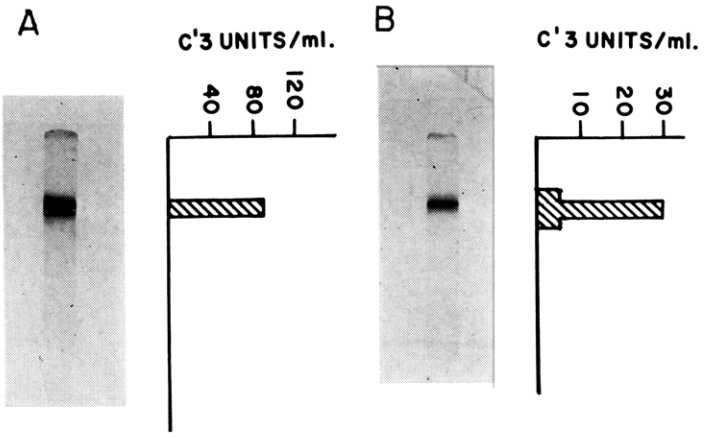

FIGURE 3: Disc electrophoresis of purified C'3 in polyacrylamide gel. Run A: small pore gel contained 3.5% acrylamide and 0.1% bisacrylamide. C'3 (50 μg) was used and gel was sliced into 3-mm sections. Run B: small pore gel contained 7% acrylamide and 0.2% bisacrylamide. C'3 (20 μg) was used and the gel was sliced into 2.5-mm sections. In matching C'3 activity and stained band, correction was made to compensate for the expansion of small pore gel during staining and destaining. Anode at the bottom.

In studies of the reaction mechanism of the complement system it is advantageous and desirable to employ highly purified reagents because the design of simple and easily interpretable experiments is thereby facilitated. As set forth in the accompanying paper, we have used the purified C'3 for studies of the kinetics of its reaction with SAC'4,2a. In addition, we have begun a chemical study of the fate of the C'3 molecule in its reaction with SAC'4,2a. As reported by us recently, SAC'4,2a cleaves C'3 into several fragments which differ in molecular size and electrophoretic mobility

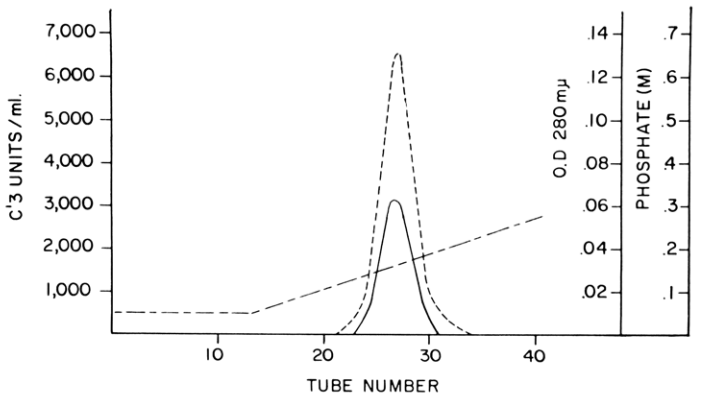

FIGURE 4: Hydroxylapatite chromatography of purified C'3. Solid line represents C'3 activity (units per milliliter); dots and dashes represent phosphate concentration; dotted line represents absorbance at 280 mμ.

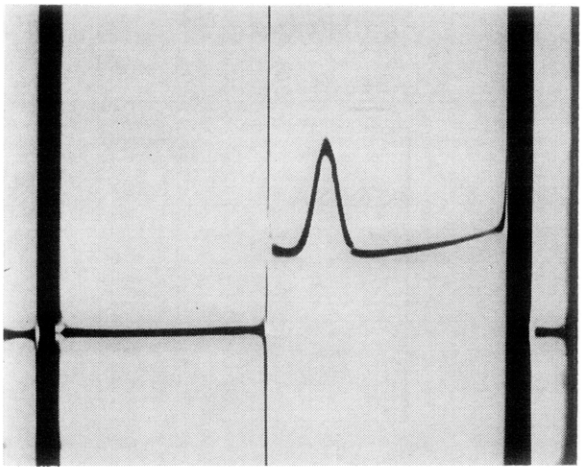

FIGURE 5: Ultracentrifugation of purified C′3. 0.4% C′3 in 0.005 M phosphate buffer (pH 7.5) containing 0.15 M NaCl. 47,600 rpm at 20°. Picture taken at 24 min. At the end of the run (64 min) there still was only one symmetrical peak.

(Mayer *et al.*, 1967). We have also started studies on the structure of C′3 and of the relationship between molecular structure and function.

Acknowledgment

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