

THE SECOND COMPONENT OF HUMAN COMPLEMENT:
ITS ISOLATION, FRAGMENTATION BY C'1 ESTERASE,
AND INCORPORATION INTO C'3 CONVERTASE*, ‡

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In the course of a comprehensive investigation of the mode of action and the biological activities of human complement, it became necessary to isolate the second component (C'2). Recently, accumulated evidence indicated that C'2 and the fourth component (C'4) fulfill their function in the complement reaction by forming a bimolecular complex which constitutes the activating enzyme for the third component (C'3) (1). Further verification of this concept required that C'2 and C'4 were available as purified proteins. Since C'2 had not been isolated so far, a method for its purification was elaborated and C'2 was identified and characterized as an individual protein. To facilitate detection of C'2 protein following its reactions with other complement components a radioactive label was introduced into the purified protein and a monospecific antiserum to C'2 was prepared. With these tools studies were initiated to determine the fate of the C'2 molecule in the complement reaction.

Materials and Methods

Serum and Pseudoglobulin.—Human blood from four donors was allowed to clot for 4 hr and the clot was removed by centrifugation. The serum in a volume of approximately 800 ml was dialyzed for 48 hr against 3×10 liters of an 8×10^{-3} M solution of Na_3EDTA of pH 5.4 and a conductance of 1.25 millimhos/cm to precipitate the euglobulins. The pseudoglobulin fraction was separated from the euglobulin fraction by centrifugation twice at 4°C and 1500 g for 30 min, then dialyzed overnight against phosphate buffer, pH 6.0, T/2 (ionic strength) = 0.06, containing 2×10^{-3} M EDTA. This buffer, which had a conductance of 4.0 millimhos/cm will hereafter be referred to as "starting buffer." A conductance of 4.0 millimhos/cm was required when the capacity of the carboxymethyl (CM)-cellulose was 0.7 meq/g. Even a slight change in absorbency for the cellulose necessitated a change in conductance for the starting buffer (see below).

Treatment of Pseudoglobulin with Diisopropylfluorophosphate (DFP).—The pseudoglobulin was incubated in a tightly stoppered Erlenmeyer flask at 37°C for 1 hr with DFP at a final

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concentration of 10^{-3} M. The DFP was obtained as a 5 M solution. Before use it was diluted with an equal volume of anhydrous isopropyl alcohol, a measure designed to reduce the volatility of this highly toxic reagent (2). Despite this measure, great care was taken to ensure that following the period of incubation at 37°C, the flask was cooled to 0–4°C before it was unstoppered.

CM-Cellulose Chromatography.—The DFP-treated material was applied to a 7.5×60 cm column containing approximately 2000 ml of packed CM-cellulose which had been equilibrated overnight with starting buffer. Following the application of the sample, 10 liters of starting buffer were passed through the column to remove proteins which, under these conditions, were not adsorbed to the CM-cellulose. C'2 together with other basic proteins were eluted by the application of a salt gradient from 0–0.7 M sodium chloride. A 2 liter beaker which served as the mixing chamber and contained 2000 ml of starting buffer was connected by siphon to a 2 liter Erlenmeyer flask containing approximately 1900 ml of 0.7 M NaCl in starting buffer. The flow rate was adjusted to 2 ml/min and 20-ml fractions were collected. The fractions containing C'2 hemolytic activity were pooled and concentrated to 20 ml by ultrafiltration in an Amicon concentration device using a UM 1 ultrafilter. This sample was again treated with DFP as outlined above.

It was found that the conductivity required for the starting buffer was highly dependent on the capacity of the CM-cellulose used. It was therefore necessary to establish the required conductivity of the starting buffer for each new batch of CM-cellulose. In a preliminary experiment the conductance of the starting buffer was lowered to 3.5 millimhos/cm and C'2 was eluted by an NaCl gradient in the usual manner. The conductivity was then determined at which the C'2 started to elute from the column, and in future experiments with the same batch of cellulose, the conductance of the starting buffer was adjusted to be 0.5 millimhos/cm below this figure.

Pevikon Block Electrophoresis.—The concentrated DFP-treated chromatography fraction was divided into two 10-ml samples, each of which was subjected to Pevikon block electrophoresis by the method previously described (3). Phosphate buffer, pH 6.0, $T/2 = 0.05$, containing 2.5×10^{-3} M EDTA was used. The polyvinyl sheeting used for the base and cover of the block was washed with 5×10^{-3} M EDTA before use. Electrophoresis was allowed to proceed for 23 hr at 4°C employing a potential gradient of 4 v/cm.

Hydroxyl Apatite Chromatography.—Since commercially available OH-apatite was found to give consistently very poor recovery of protein, hydroxyl apatite was used which was prepared following the method of Tiselius et al. (4). New batches of freshly prepared hydroxyl apatite also gave a very low recovery of C'2 hemolytic activity. However, by passing 200 mg of human serum albumin through the packed OH-apatite column before application of the C'2 preparation, this problem was alleviated. It was found unnecessary to repeat this procedure when the column was reused. The material from the two Pevikon blocks was contained in approximately 100 ml and this volume was applied to a 16×1.5 cm column of OH-apatite which had been equilibrated with phosphate buffer, pH 5.8, conductance 3 millimhos/cm. Following application of the sample, the column was washed with 100 ml of the same buffer, followed by 250 ml of each of four buffers having the same pH but different conductivities. The concentration of phosphate buffer was increased in a step-wise manner to yield conductances of 12, 14, 16, and 17 millimhos/cm, respectively. Finally, in order to elute residual protein, the column was washed with phosphate buffer having a conductance of 68 millimhos/cm. The flow rate of the column was adjusted to 1 ml/min and 4-ml fractions, were collected. Since at this stage the protein concentration in the column effluent was low, it was necessary to use siliconized tubes for collection of the samples in order to preserve C'2 activity.

Concentration of Purified C'2.—The fractions containing C'2 activity were pooled, and to protect C'2 during the subsequent concentration procedure, monomeric, carboxymethylated

human serum albumin was added in an amount sufficient to yield 1 mg/ml in the concentrated pool. The final material was divided into 0.1 or 0.2 ml aliquots contained in 1×7 cm siliconized tubes and was frozen in liquid nitrogen and stored at -70°C until used.

Preparation of Human Serum Albumin as Carrier Protein for Purified C'2.—1 g of crystallized human serum albumin (Behringwerke AG, Marburg/Lahn, Germany) dissolved in 10 ml of veronal buffer, pH 8.6, $T/2 = 0.05$, was subjected to Pevikon block electrophoresis for 16 hr. The peak fractions of the major component were pooled and treated overnight at 4°C with iodoacetamide at a final concentration of 10^{-2} M. Following concentration, this material was applied to a Sephadex G-100 column and the monomeric form of the iodoacetamide-treated albumin was isolated and concentrated.

Preparation of Complement Components and of Erythrocyte-Antibody-Complement Complexes.—Partially purified C'1 was prepared in its macromolecular form according to Nelson (5). The esterase moiety of the first component (6) was isolated according to Haines and Lepow (7). Highly purified C'3 and C'4 were isolated according to the methods previously described (8, 9). C'2 was oxidized by treating 1 vol of C'2 with an equal volume of 5×10^{-5} M I_2 in 2.5×10^{-3} M KI, as described in detail elsewhere (10). The C'2 hemolytic activity assay (10) used in this study and the preparation of the assay cells, EAC'1a, 4, have been described previously (11).

Polyacrylamide Gel Electrophoresis.—For disc electrophoresis on polyacrylamide gel the method of Davis (12) was used, employing a concentration of acrylamide in the separating gel of 6%.

In order to locate C'2 hemolytic activity to a protein band in the acrylamide gel, duplicate gels were employed. One was stained for protein and the other was cut into 2-mm segments each of which was transferred to a 1×7 cm tube containing 0.2 ml of phosphate buffer, pH 6 and 0.005 M EDTA. The gel segments were ground with a glass rod and after 1 hr of standing at 0°C , the tubes were centrifuged at 0°C for 30 min and the supernatants were removed with a Pasteur pipette and tested for C'2 hemolytic activity. In experiments in which C'2- ^{125}I was utilized, the radioactivity of each gel segment was measured before extraction of the protein.

Radioactive Labeling of C'2.—C'2 was labeled with ^{125}I by the method of McConahey and Dixon (13). In order to preserve the hemolytic activity of C'2 during the labeling process it was found necessary to reduce the amount of the oxidizing agent, chloramine T, to $5 \mu\text{g}/\text{mg}$ of protein. Since the albumin which was used to stabilize and protect highly purified C'2 was also labeled with radioactive iodine, it was necessary to replace the albumin- ^{125}I by non-radioactive albumin. The labeled preparation was extensively dialyzed against 2×10 liters of phosphate buffer, pH 6.0, $T/2 = 0.1$, containing 0.005 M EDTA, followed by dialysis against 10 liters of the starting buffer used for CM-cellulose chromatography (see above). The sample was then applied to CM-cellulose contained in a 10 ml pipette previously equilibrated with starting buffer. Under these conditions, the albumin- ^{125}I passed through the CM-cellulose, while the C'2- ^{125}I was adsorbed, and after the albumin- ^{125}I had been washed from the column with starting buffer, the C'2- ^{125}I was eluted by an NaCl gradient. The C'2- ^{125}I was collected into siliconized tubes, pooled and concentrated after addition of unlabeled, iodoacetamide treated, monomeric albumin. To prepare ^{125}I -C'2 from C'2- ^{125}I , 2×10^{-5} M I_2 in 1×10^{-3} M KI was employed.

Sucrose Density Gradient Ultracentrifugation.—Linear sucrose density gradients (7–31%) were prepared with the Buchler automatic density gradient device. Sucrose was dissolved in phosphate buffer, pH 6.0 $T/2 = 0.1$, containing 0.1 M NaCl. Ultracentrifugation was performed for 15 hr at 50,000 rpm and 4°C in a Spinco L-2 machine employing an SW 50 rotor. Fractions were collected using the Buchler gradient fractionation device. For s rate determinations, the following reference substances were used: human γG -globulin- ^{131}I (7S), human hemoglobin (4.5S), and equine cytochrome C (1.7S).

Gel Filtration.—For estimation of the diffusion coefficient of C'2, a 105×2.5 cm column, Sephadex G-200 and phosphate buffer, pH 6.0, $T/2 = 0.1$, containing 0.1 M NaCl were employed. For application, the density of the sample was increased with NaCl and layered between the gel bed and the supernatant buffer. 1-ml fractions were collected. As reference substances with known diffusion coefficients were employed: human γ G-globulin- ^{181}I (3.8×10^{-7} cm²/sec), human hemoglobin (6.8×10^{-7} cm²/sec), and equine cytochrome C (13.0×10^{-7} cm²/sec).

Preparation of Specific Antiserum of Human C'2.—0.1 ml of a mixture containing equal volumes of the purified C'2 preparation and Freund's complete adjuvant was injected into each popliteal lymph node of two rabbits, according to the method of Goudie et al. (14). 1 month later 0.1 ml of a similar mixture was injected intramuscularly into each of two sites in the hindquarters of both rabbits. 6 days later the animals were bled and the antiserum was adsorbed with human serum albumin and γ G-globulin.

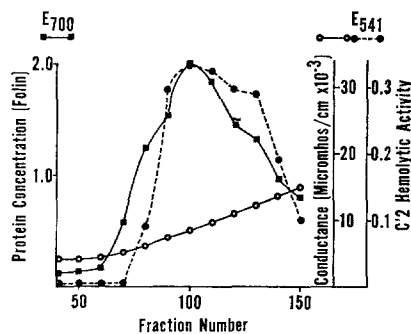


FIG. 1. First step of C'2 purification: Elution from CM-cellulose. C'2 hemolytic activity together with 2–4% of the pseudoglobulin are eluted by an NaCl concentration gradient started at tube 1. 96–98% of the protein was washed through the column with the starting buffer (not shown) before application of the gradient. Fractions 80–150 were pooled and concentrated for further separation by Pevikon block electrophoresis.

RESULTS

Essential Measures for Preservation of C'2 Hemolytic Activity during and after Purification.—Since it was found that C'2 activity was most stable at pH 6 and in the presence of EDTA, most manipulations were performed at these conditions. Despite these measures, substantial inactivation of C'2 was encountered during CM-cellulose chromatography and Pevikon block electrophoresis. On investigation, it was found that a substance with the ability to inactivate C'2 (and C'4) was present in the effluent from the CM column in some of the fractions containing C'2 hemolytic activity (Fig. 1). On pooling the C'2-containing fractions, this substance, which is probably identical with C'1 esterase, was introduced into the preparation and caused inactivation of C'2 encountered at this stage and during the subsequent concentration step and Pevikon block electrophoresis. The C'2 which was recovered from the Pevikon block in hemolytically active form remained stable throughout periods of

storage of several months. It was therefore concluded that the inactivating enzyme was separated from the C'2 by electrophoresis. Since, like C'1 esterase (6), this enzyme was inactivated by DFP, both the pseudoglobulin and the C'2 pool from the CM-cellulose column were subjected to DFP treatment. Subsequent to the introduction of this measure no inactivation of C'2 has occurred during the isolation procedure.

In the final purification step C'2 is obtained in a concentration of 5–10 $\mu\text{g}/\text{ml}$. Concentration of this dilute protein solution by ultrafiltration pressure dialysis or lyophilization resulted in total loss of the C'2 hemolytic activity. Inactivation

TABLE I
Recovery and Specific Hemolytic Activity of C'2 during Isolation Procedure

Preparation No.	Purification step	Volume	Protein*	Effective molecules	Specific hemolytic activity
		<i>ml</i>	<i>mg</i>		<i>(eff. mol./μg)</i>
67	Whole serum	720	78,000	(1.3×10^{14})	(1.67×10^6)
	Pseudoglobulin	710	69,000	(1.42×10^{14})	(2.06×10^6)
	CM-effluent (unconcentrated)	530	1,380	4.2×10^{13}	3.04×10^7
	CM-effluent (concentrated)	11.5	1,400	2.6×10^{13}	1.86×10^7
	Pevikon block	137	13.8	1.3×10^{13}	9.4×10^8
	OH-apatite (concentrated)	6	2.88	5.6×10^{12}	1.94×10^9
70	Whole serum	840	72,000	(1.18×10^{14})	(1.64×10^6)
	Pseudoglobulin	820	59,000	(1.15×10^{14})	(1.95×10^6)
	CM-effluent (unconcentrated)	850	2,720	3.4×10^{13}	1.26×10^7
	CM-effluent (concentrated)	19	2,600	2.45×10^{13}	9.4×10^6
	Pevikon block	311	25	3.5×10^{12}	1.4×10^8
	OH-apatite (concentrated)	6	2.5	3.51×10^{12}	1.4×10^9

* Based on Folin method.

was attributed to denaturation of C'2 in dilute solution. Concentration was therefore attempted in the presence of a carrier. Both pressure dialysis and lyophilization in the presence of 10^{-3} M glycine resulted in 90% loss of the hemolytic activity. However, no loss of activity was encountered when C'2 was concentrated by pressure dialysis in the presence of human serum albumin. As the material was to be tested for purity on polyacrylamide gels, and as the polymeric form of albumin migrated more slowly than the monomer and could obscure contaminants in the C'2 preparation, the monomeric form of the carboxymethylated albumin was isolated and then used in all subsequent preparations as carrier protein.

Partial Purification of C'2 by CM-Cellulose Chromatography and Preparative Electrophoresis.—Under the conditions employed, only 2–4% of the DFP-treated pseudoglobulin applied to the CM-cellulose column was adsorbed, the remaining 96–98% was washed through by the starting buffer. The adsorbed protein was eluted by a salt concentration gradient (Fig. 1). In terms of protein, a 30-fold purification was thus achieved which, however, was accompanied by a three- to fourfold loss of hemolytic activity as measured by effective molecule titration (Table I).

The material from the CM-cellulose column which contained C'2 activity was concentrated and further separated by Pevikon block electrophoresis. At pH

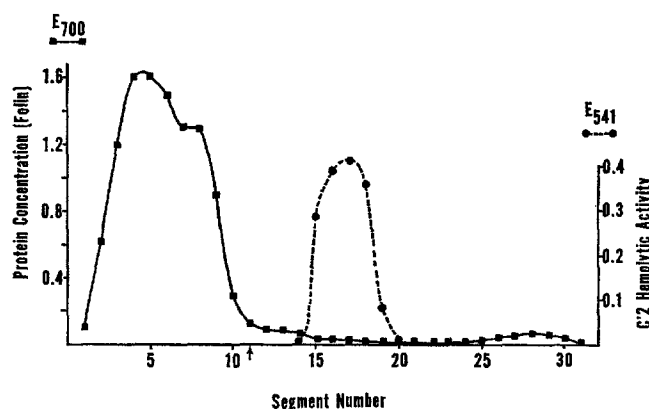


FIG. 2. Second step of C'2 purification: Pevikon block electrophoresis performed in phosphate buffer, pH 6, $T/2 = 0.05$, containing 2.5×10^{-3} M EDTA. Origin is indicated by the arrow; the anode is to the right. Fractions 15–19 were pooled for further separation by hydroxyl apatite chromatography.

6, C'2 migrated towards the anode together with contaminating β -globulins such as C'3, C'4, and C'5. γ -Globulin which represented 99% of the material moved towards the cathode (Fig. 2). While 2 g of protein was applied to two Pevikon blocks, approximately 20 mg of protein was recovered from the β -globulin region which contained C'2 hemolytic activity.

Final Purification of C'2 by Hydroxyl Apatite Chromatography.—Many different methods were explored, such as: chromatography on CM-cellulose and TEAE-cellulose; gel filtration on Sephadex G-200; preparative electrophoresis on polyacrylamide gel and agar; hydroxyl apatite chromatography under alkaline conditions. None of these methods proved entirely satisfactory, since only partial purification resulted from each. Finally, the method selected which resulted in total purification of C'2 and its identification as a discrete serum protein was chromatography on hydroxyl apatite under acid conditions, using a stepwise elution procedure.

At pH 5.8, C'2 was eluted from the column at 16 millimhos/cm and emerged as a discrete protein peak which corresponded with the peak of C'2 hemolytic activity (Fig. 3). With some batches of hydroxyl apatite, C'2 was eluted from the column at 15 millimhos/cm. In such cases, a similar elution pattern was obtained when the buffers with conductances of 14 and 16 millimhos/cm were substituted by buffers having conductances of 13.2 and 15 millimhos/cm, respectively.

Identification of C'2 with a Discrete Serum Protein.—On analysis of the purified C'2 by polyacrylamide gel electrophoresis, two protein bands were demonstrable which were of unequal intensity and which were located rather

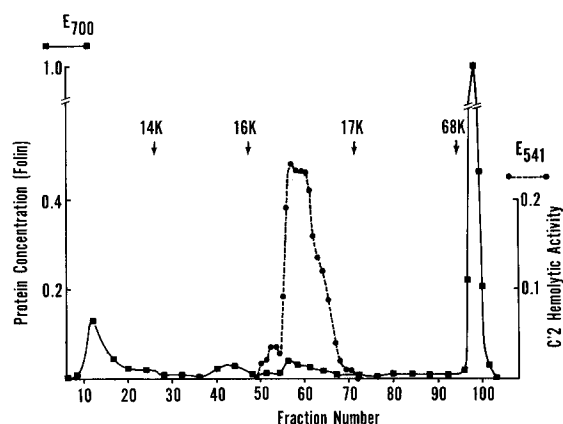


FIG. 3. Final step of C'2 purification: Chromatographic separation on hydroxyl apatite. The protein (fractions 15–19, Fig. 2) was applied in phosphate buffer, pH 5.8, 3 millimhos, and was eluted stepwise by buffers of increasing phosphate concentration. The first peak shown was eluted at 12 millimhos and the arrows mark the start of each successive step. Fractions 54–66 were concentrated in the presence of human serum albumin (see text).

close to each other. C'2 hemolytic activity recovered from the gel corresponded exactly to the major, more slowly migrating protein disc (Fig. 4). There was no hemolytic activity associated with the minor and somewhat faster migrating component. As will be shown below (Fig. 10), inactivation of the isolated C'2 by C'1 esterase resulted in complete disappearance of the protein band corresponding to hemolytically active C'2. The inactive protein, C'2i, appeared as a single disc in a more anodal position which corresponded directly to that of the minor component present in untreated C'2. This observation, as well as immunochemical analyses (see below) indicated that the minor component in purified C'2 constitutes C'2i and not an unrelated contaminant. The proportion of C'2i present in each of 10 preparations of C'2 varied between 5 and 50% of the total protein. In each case, treatment with C'1 esterase resulted in total

conversion of the active C'2 protein to the inactive, faster migrating product, C'2i.

Correlation between C'2 Hemolytic Activity and C'2-¹²⁵I Protein.—Under the conditions employed for radioactive labeling, uptake of ¹²⁵I by C'2 was low, not

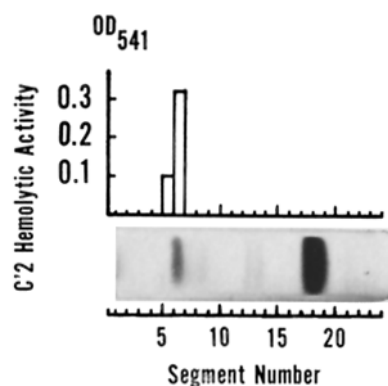


FIG. 4. Correlation between C'2 hemolytic activity and protein upon polyacrylamide gel electrophoresis. Anode is towards the right. Duplicate gels were employed, one was stained for protein, the other was analyzed for C'2 hemolytic activity. Protein bands in segments 13, 14, 18, and 19 represent the carrier protein, human serum albumin (see Fig. 10).

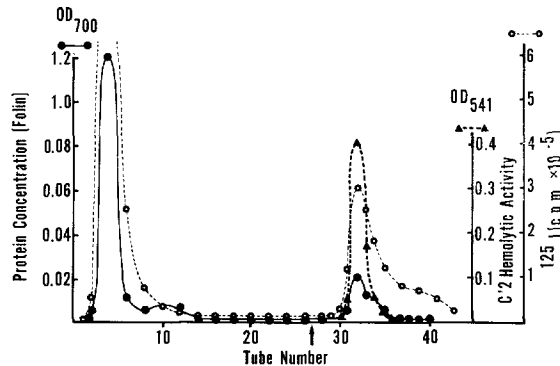


FIG. 5. Chromatographic separation of albumin-¹²⁵I from C'2-¹²⁵I, and correlation of C'2 protein, hemolytic activity, and radioactive label. The arrow marks application of the NaCl concentration gradient. Fractions 31–34 were pooled and concentrated in the presence of unlabeled human serum albumin.

exceeding 0.6%. The specific radioactivity was between 10,000 and 13,000 cpm/ μ g. That the label was definitely incorporated into C'2 was shown by two different methods. Chromatography of the carrier albumin-purified C'2 mixture on CM-cellulose revealed an excellent correlation between the distributions of C'2 hemolytic activity, C'2 protein, and radioactivity (Fig. 5). When

C'2- ^{125}I , following separation from albumin- ^{125}I , was subjected to polyacrylamide gel electrophoresis, radioactivity was found to be associated with both protein bands. The major peak of radioactivity was contained in the segment from which the hemolytically active C'2 was eluted. Following treatment of the labeled preparation with C'1 esterase, loss of C'2 hemolytic activity was associated with a characteristic shift of the radioactivity peak to the more anodal position of C'2i.

Yield and Specific Hemolytic Activity of Purified C'2.—As can be seen from the values listed in Table I, the yield of C'2 in terms of effective molecules (15) was 3–4% and in terms of protein approximately 0.0035%. Hence, there was a 1000-fold increase in activity-protein ratio. From this figure as well as from the recovery data it may be inferred that the concentration of C'2 in whole human serum is one thousandth of the serum protein concentration; i.e., approximately

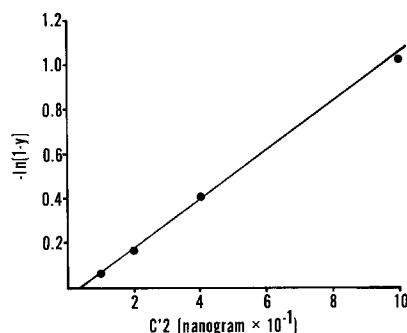


FIG. 6. Dose response for highly purified C'2: Average number of C'2a sites per cell ($-\ln(1-y)$) versus nanograms of C'2.

70 $\mu\text{g}/\text{ml}$. This figure must be regarded as a maximal estimate, the true value being probably lower than this. The uncertainty stems from the consideration that effective molecule titrations of C'2 in whole serum may be falsely high since other complement components present in serum are likely to enhance the hemolytic assay system. Secondly, the variable proportion of C'2i in the preparations tends to decrease the activity-protein ratio of purified C'2. Therefore, the true degree of purification achieved in the isolation of C'2 might be two or three times greater than indicated in Table I, and thus the true serum concentration of C'2 only half or one-third of the calculated value, probably in the range of 15–30 $\mu\text{g}/\text{ml}$.

With isolated C'2 it became possible to determine directly the number of C'2 molecules needed to produce one "effective molecule" in the sense of the one-hit theory of immune hemolysis (16). A typical dose response curve is shown in Fig. 6, where the amount of C'2 is expressed in absolute weight units and where per cent hemolysis was converted to the negative natural logarithm of the fraction of unlysed cells, i.e. $-\ln(1-y)$; y being the fraction of cells

which were lysed. In three experiments, each using a different preparation of C'2, it was found that an average of 1000 C'2 molecules equals one "effective molecule;" i.e., the hemolytic efficiency of C'2 is approximately 0.1%. It is emphasized that these calculations are based on the C'2 protein which was fully convertible by C'1 esterase.

Molecular Weight Determination.—For the determination of the sedimentation coefficient, C'2-¹²⁵I was subjected to ultracentrifugation in a sucrose density gradient. Using three reference proteins (Fig. 7) the sedimentation velocity of C'2 was found to be 5.2S. The diffusion coefficient (D) was estimated by gel filtration on Sephadex G-200 according to the method of Andrews (17). Using the same three reference proteins (Fig. 8), the diffusion coefficient of

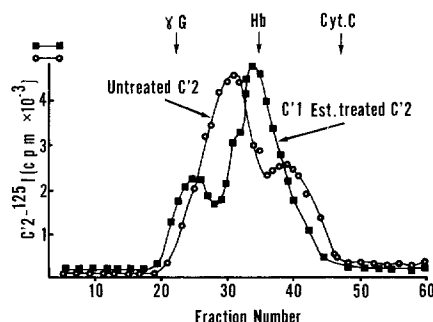


FIG. 7. Comparative ultracentrifugal analysis of C'2 and C'2 following treatment with C'1 esterase for 30 min at 37°C, performed in a 7–31% sucrose density gradient in phosphate buffer, pH 6, $T/2 = 0.1$, containing 0.1 M NaCl; for 16 hr at 50,000 rpm. As reference substances were used: human γ G-globulin (γ G); human hemoglobin (Hb); and equine cytochrome C. (Cyt. C). Sedimentation is toward the left.

C'2 was found to be 4.0×10^{-7} cm²/sec. Assuming the partial specific volume of C'2 to be 0.73 and utilizing the above recorded values for s and D , the molecular weight of C'2 was calculated to be 117,000, and the frictional ratio (18) to be 1.6. Both the ultracentrifugal and filtration analyses of untreated C'2 revealed a second, minor component which apparently was of smaller molecular size than the main component.

Immunochemical Studies of Purified C'2 and of C'2 in Serum.—Purified C'2 gave a strong positive reaction in Ouchterlony plates with anti-C'2, and negative reactions with anti-C'3, anti-C'4, and anti-C'5. Tests with anti-transferrin and antihemopexin were also negative. However, an anti- γ G-globulin produced a weak precipitin reaction in the immediate vicinity of the antigen well, indicating the presence of a small amount of γ G-globulin.

On immunoelectrophoretic analysis purified C'2 reacted only with anti-C'2: a single, well-defined precipitin arc was seen (Fig. 9). In agarose C'2 travelled

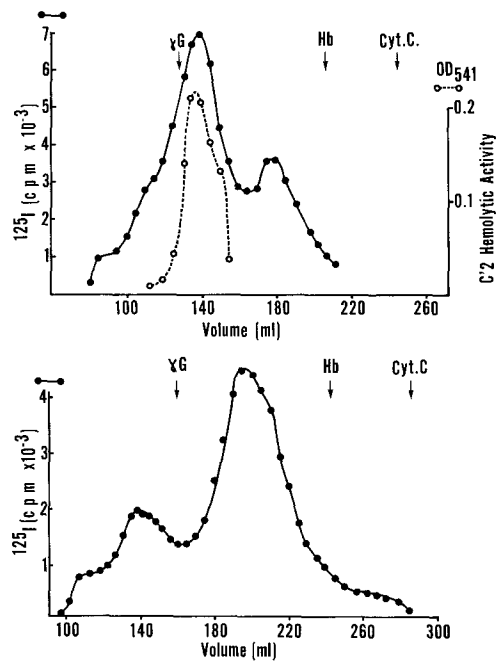


FIG. 8. Elution patterns obtained following gel filtration of C'2 on a Sephadex G-200 column (105×2.5 cm). The upper pattern shows untreated C'2; the lower, C'2 following treatment with C'1 esterase for 30 min at 37°C . The columns were equilibrated with phosphate buffer, pH 6, $T/2 = 0.1$, containing 0.1 M NaCl . Each sample contained three reference substances: human γG -globulin (γG); human hemoglobin (Hb); and equine cytochrome C (Cyt. C).

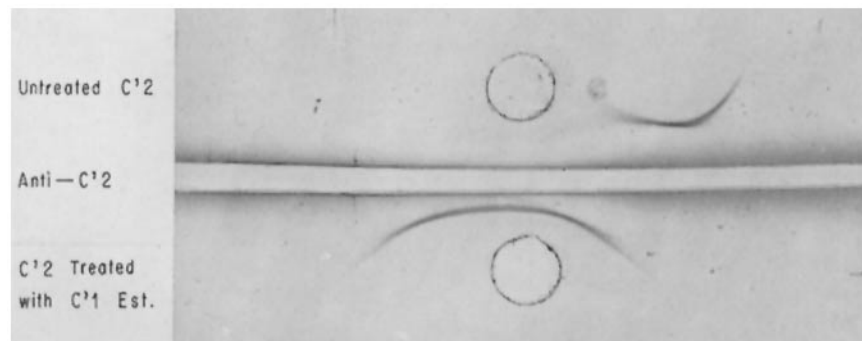


FIG. 9. Immunoelectrophoretic analysis of C'2 (upper well) and C'2 following treatment for 30 min at 37°C with C'1 esterase (lower well). Antibody produced against purified C'2 is in the center trough. Anode is to the right.

as a β -globulin, as was expected on the basis of its mobility on block electrophoresis. However, in agar its mobility was much increased and only slightly smaller than that of albumin. This differential behavior of C'2 in dependence of the type of gel used was highly reproducible. C'2 hemolytic activity could be eluted from agar as well as from agarose slides and its distribution corresponded in both media exactly to that of the C'2 precipitin arc.

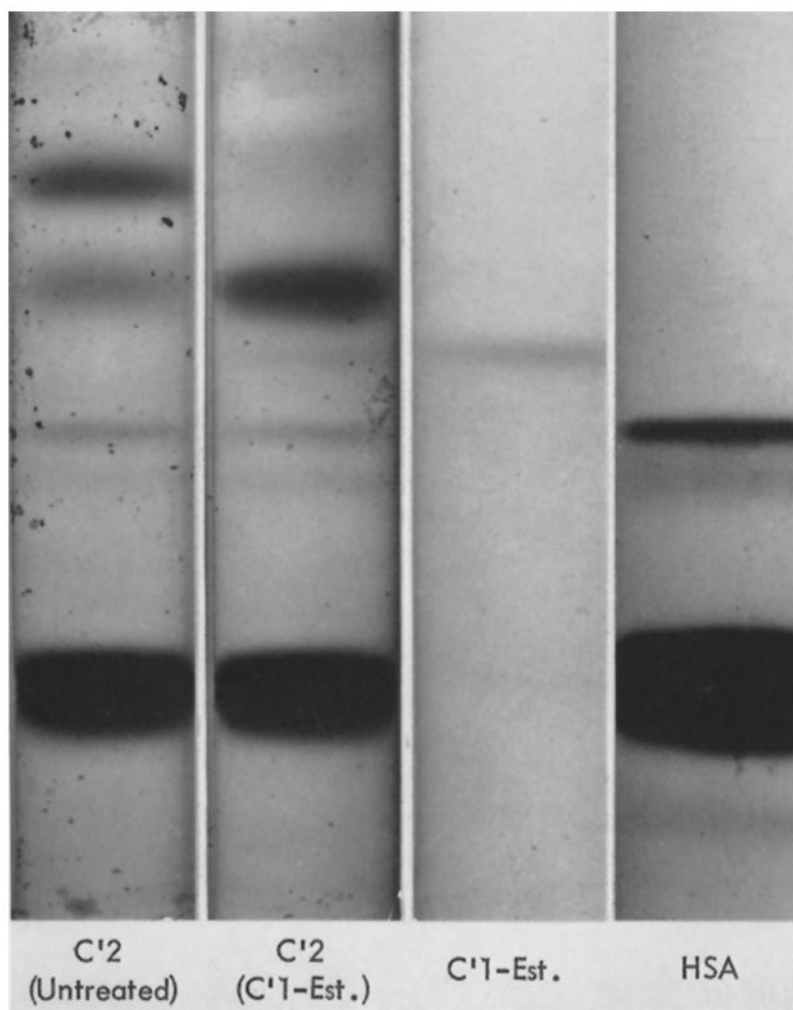


FIG. 10. Comparative polyacrylamide gel electrophoresis analysis of untreated C'2 and C'2 following incubation with C'1 esterase for 30 min at 37°C. Also shown are analyses of C'1 esterase alone and of the carrier protein, human serum albumin. Anode is towards the bottom.

When whole serum was subjected to electrophoresis in agar, the mobility of C'2 was reduced to that of a fast β -globulin. The same reduction in mobility was observed with purified C'2 following the addition of purified C'4. It is suggested, therefore, that the slower mobility of C'2 on agar electrophoresis of serum is due to the previously demonstrated reversible complex formation between C'2 and C'4 (1).

Effect of C'1 Esterase on Purified C'2.—Treatment of C'2 with C'1 esterase for 30 min at 37°C results in marked physicochemical alteration of the C'2 molecule which coincides with loss of its hemolytic activity. Compared with native C'2 the inactive reaction product C'2i moves more slowly on agar gel electrophoresis and immunoelectrophoresis shows the C'2i arc to be wider than the C'2 arc, indicating either greater diffusibility or electrophoretic heterogeneity of the product (Fig. 9).

As shown in Fig. 10, disc electrophoresis revealed C'2i in a more anodal position than untreated C'2. It also demonstrated clearly that all native C'2

TABLE II
Molecular Parameters of C'2 and C'2i

Protein	<i>s</i>	D	f/f ₀	Molecular weight
C'2	5.2	4.0	1.6	117,000*
C'2i	4.5	4.9	1.4	83,000†

* Mean of five experiments.

† Mean of three experiments.

molecules are susceptible to the action of C'1 esterase and can be converted to C'2i. That the faster migrating, minor component present in purified C'2 (Figs. 4 and 10) represents C'2i was demonstrated immunochemically. Preparations which contained this component also showed the slow conversion product on immunoelectrophoresis. When unstained polyacrylamide gels were embedded into agar and then analyzed with anti-C'2 by diffusion in agar gel, a bimodal precipitin line was seen with untreated, purified C'2, clearly showing that the minor component was antigenically closely related to the major protein component.

Although yielding only one single disc on polyacrylamide electrophoresis, C'1 esterase-produced C'2i was found to be heterogeneous by both ultracentrifugation (Fig. 7) and gel filtration (Fig. 8). For these experiments C'2-¹²⁵I was used. The major component in C'2i had an *s* rate of 4.5 and a diffusion coefficient of 4.9×10^{-7} cm²/sec and therefore a calculated molecular weight of 84,000 and a frictional ratio of 1.4. In two additional analyses molecular weights of 82,000 and 81,000 were obtained. Comparing these values with the molecular parameters of untreated C'2 (Table II) they indicate that C'1 esterase effects fragmentation of the C'2 molecule.

Demonstration of the Physical Incorporation of C'2-¹²⁵I into C'3 Convertase — On the basis of molecular weight determinations it was previously postulated that the enzyme C'3 convertase is a protein-protein complex which is derived from C'2 and C'4 (1). Utilizing C'2-¹²⁵I this concept could be further examined. The enzyme was generated from C'2-¹²⁵I and monomeric C'4i (19) by the action of C'1 esterase essentially as outlined before (1). The reaction mixture

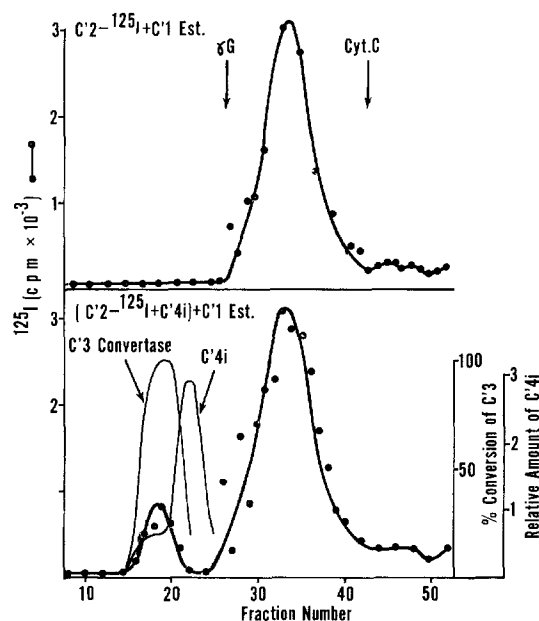


FIG. 11. Demonstration of incorporation of C'2 into C'3 convertase by sucrose density gradient ultracentrifugation: C'2-¹²⁵I treated with C'1 esterase in the absence (upper) and presence (lower) of C'4i. Ultracentrifugation performed under identical conditions to those described in the legend to Fig. 7. Human γ G-globulin (γ G) and equine cytochrome C (Cyt. C) were used as reference substances. C'4i was quantitated by the distance between the antigen well and the precipitin arc formed with anti-C'4 when tested by Ouchterlony analysis. C'3 convertase activity was determined by conversion of C'3 to C'3i.

was then subjected to sucrose density gradient ultracentrifugation and the distribution of radioactivity was compared with that observed in the control which consisted of C'2-¹²⁵I treated with C'1 esterase in the absence of C'4i. The results are depicted in Fig. 11. A fast sedimenting radioactivity peak was observed which corresponded exactly to the position of C'3 convertase activity and which was not seen in the control that lacked C'3 convertase activity. There was also a small proportion of C'4i which sedimented faster than the bulk of C'4i and this material too was distributed in the region of C'3 convertase

activity. As expected, the main portion of C'2-¹²⁵I sedimented after the reaction as unbound C'2i with an *s* rate of 4.5S. This experiment constitutes a direct physical demonstration of the incorporation of the C'2 molecule, or a part thereof, into the enzyme C'3 convertase.

DISCUSSION

The second component of human complement is a trace constituent of serum which in the past has escaped detection as a protein, being recognized solely through its biological activity. The present paper gives the first detailed description of a method which permits isolation of C'2 from human serum in a high degree of purity and in hemolytically active form. This method has been elaborated, modified and tested in this laboratory during the past 3 yr and has been found to produce preparations of consistent quality. In absolute terms the yield of purified C'2 is low, however, if it is assumed, as is probable, that the serum concentration of C'2 is approximately 20 µg/ml, the recovery is greater than 10%.

Purification of human C'2 has been reported in abstract form by Lepow et al. (20), simultaneous with the first brief report on the subject from this laboratory (21). Since no further account has been rendered by these workers, a comparison of the two methods and of the purity of the final products is not possible. Guinea pig C'2 has been functionally purified by Borsos et al. (22) and by Nelson et al. (5). Although a useful reagent, functionally pure C'2 may consist of a mixture of many serum proteins as revealed by immunochemical analysis (23). Employing an as yet unpublished biological procedure, Mayer (23) apparently achieved a very high degree of purification of guinea pig C'2. Exploiting the specific affinity of C'2 for C'4, he first adsorbed C'2 from serum to EAC'4 cells at low ionic strength and then eluted it from the cells at physiological ionic strength.

Using the C'2 purified according to the method described in this paper, it was possible to characterize C'2 by disc electrophoresis as a discrete protein: C'2 activity corresponded to a single, well-defined protein band and, as theoretically required, this protein was totally convertible by C'1 esterase, the converting enzyme of C'2. As anticipated, the specific hemolytic activity of purified C'2 was very high, 1 µg contained 2×10^9 effective molecules. Nevertheless, approximately 1000 potentially effective C'2 molecules were required to produce one hemolytically effective, or site-forming molecule. This relative inefficiency has been found to be an inherent feature of the C'2 reaction mechanism as has been reported separately (24).

Monospecific antiserum to C'2 was produced by injecting minute amounts of purified protein directly into the popliteal lymph nodes of rabbits. In Ouchterlony plates this antiserum detects only one protein in whole human serum which is immunologically identical with purified C'2. Since the anti-C'2 anti-

body was present neither in several potent antisera to whole human serum nor in a variety of specific antisera to known serum proteins, it may be concluded that the protein described in this communication is distinct from previously recorded serum constituents. The immunochemical definition of C'2 with monospecific anti-C'2 has assumed special significance in analyzing the biochemical defect of genetically C'2-deficient individuals. These studies will be reported elsewhere.¹

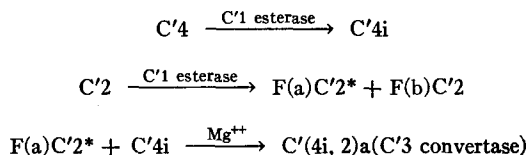
Incorporation of radioactive iodine into the C'2 molecule without loss of hemolytic activity afforded working with amounts too small to be measured reliably by chemical analysis. The radioactive label as well as the specific antibody made possible the detection of hemolytically inactive C'2 and thus an analysis of the fate of C'2 in its reaction with C'1 esterase and C'4. Admittedly, these latter studies are very incomplete, as they have been hampered by lack of sufficient material. Nevertheless, it has been possible to demonstrate clearly by four different methods (ultracentrifugation, gel filtration, disc and immunoelectrophoresis) that C'2 undergoes characteristic physicochemical changes when acted upon by C'1 esterase. These experiments suggest that in addition to changes in electric charge, there occurs a reduction in molecular size; i.e., fragmentation of the molecule. Molecular weight estimates of native and C'1 esterase-treated C'2 consistently revealed a difference of approximately 35,000. Yet three replicate experiments failed to give convincing evidence for a fragment of this size (Figs. 7 and 8). In addition to the 83,000 molecular weight component, the esterase-treated C'2 contained a small amount of heavier material which must have arisen by aggregation. The possibility was considered that the aggregates were derived from the lighter fragment. However, in a fourth experiment with C'1 esterase-treated C'2, the aggregates were not observed (Fig. 11). It is also conceivable that the postulated fragment is obscured by the larger fragment from which it did not separate sufficiently in these experiments. Or it might be derived from a region of the C'2 molecule which does not become labeled with iodine. Since all experiments were carried out with C'2-¹²⁵I, it would thus escape detection. Further work is needed to clarify the C'1 esterase effect.

Taking a different methodological approach to this problem, Mayer and his colleagues (25-27) working with guinea pig C'2 arrived at conclusions similar to those drawn from the above presented results. These workers produced antiserum to a chromatographic fraction of guinea pig serum containing C'2. By absorption with complement-cell intermediate complexes they rendered it suitable for the detection of active and inactive C'2 by neutralization competition. With this immunochemical tool they found C'2 to be physically present on EAC'1a,4,2a and to be released from this complex with its decay to the EAC'1a,4 state. Since the released C'2 was of smaller molecular size than native

¹ Polley, M. J. 1968. Inherited C'2 deficiency in man. Lack of immunochemically detectable C'2 protein in serum from deficient individuals. *Science*. In press.

C'2, they postulated cleavage of C'2 by cell-bound C'1 as a necessary step in C'2 uptake.

Perhaps the most significant aspect of the present work is the direct demonstration of the incorporation of C'2 into the complement enzyme C'3 convertase. This enzyme, which cleaves C'3, has previously been shown to be derived from C'2 and C'4 by the action of C'1 esterase (1). Since the molecular weight of the enzyme (305,000) was distinctly larger than the molecular weights of either C'2 (115,000) or C'4 (229,000), it was postulated that the activity was resident in a C'2-C'4 protein-protein complex. However, since the molecular size of the enzyme was by 39,000 smaller than the sum of the molecular weights of the two precursors, it was further postulated that only a fragment of the C'2 molecule becomes complexed to C'4. The size of this fragment was estimated to be approximately 76,000. This is very close indeed to the value found in this study for the molecular weight of the main component of C'1 esterase-converted C'2. Moreover, when radioactively labeled C'2 was utilized for generation of C'3 convertase approximately 10% of the labeled material sedimented on density gradient ultracentrifugation together with the C'3 convertase activity peak. Thus the concept regarding the formation of C'3 convertase previously advanced from this laboratory (1) is further supported by the results of this study: This enzyme is apparently unique in that it is formed from two distinct precursor proteins, each being totally devoid of enzymatic activity. Activity is generated by the action of another enzyme, C'1 esterase, on both precursors, but not by C'1 esterase action on either precursor individually. Apparently the activity depends on the joining of a fragment of C'2 (F(a)C'2) in its nascent state to C'1 esterase modified C'4 according to:



Where F(a)C'2* denotes a transient state of activation of the F(a) fragment of C'2 and where F(b)C'2 is a postulated, not yet verified, smaller fragment of C'2. With purified C'2 available it will now be possible to determine the chemical nature of the C'2 cleavage, the type of bond which holds the active enzyme together, and the effect of oxidation on C'2 (10) which leads to a marked increase of activity and stability of the C'4, 2a complex.

SUMMARY

A method has been described for the purification and isolation of the second component of complement (C'2) from human serum. The protein is a β_1 -globulin with an approximate molecular weight of 117,000. Immunochemical analysis using a variety of specific antisera, including a monospecific antiserum

to the isolated protein, indicate that the C'2 protein represents a heretofore unrecognized human serum constituent. Isolated C'2 contained 2×10^9 "effective molecules" per microgram and 1000 hemolytically active C'2 molecules were required to produce a single hemolytically effective C'2 site on erythrocytes undergoing immune cytolysis. C'1 esterase treatment of C'2 resulted in reduction of both its electrophoretic mobility and its molecular size, the latter observation indicating fragmentation of the molecule. Direct evidence was presented for the physical presence of C'2 as an integral part of the enzyme C'3 convertase.

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Addendum.—Most of the commercially available batches of diisopropylfluorophosphate (DFP) were found to be partially hydrolyzed and therefore not suited for use in the isolation of C'2. Highly reactive DFP was obtained from Boots of Nottingham, England.

BIBLIOGRAPHY

1. Müller-Eberhard, H. J., M. J. Polley, and M. A. Calcott. 1967. Formation and functional significance of a molecular complex derived from the second and fourth component of human complement. *J. Exptl. Med.* **125**:359.
2. Levine, L. 1955. Inhibition of immune hemolysis by diisopropyl fluorophosphate. *Biochim. Biophys. Acta.* **18**:283.
3. Müller-Eberhard, H. J. 1960. A new supporting medium for preparative electrophoresis. *Scand. J. Clin. Lab. Invest.* **12**:33.
4. Tiselius, A., S. Hjertén, and Ö. Levin. 1956. Protein chromatography on calcium phosphate columns. *Arch. Biochem. Biophys.* **65**:132.
5. Nelson, R. A., 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.
6. Haines, A. L., and I. H. Lepow. 1964. Studies on human C'1-esterase. II. Function of purified C'1-esterase in the human complement system. *J. Immunol.* **92**:468.
7. Haines, A. L., and I. H. Lepow. 1964. Studies on human C'1-esterase. I. Purification and enzymatic properties. *J. Immunol.* **92**:456.
8. Nilsson, U. R., and H. J. Müller-Eberhard. 1965. Isolation of β_{1F} -globulin from human serum and its characterization as the fifth component of complement. *J. Exptl. Med.* **122**:277.
9. Müller-Eberhard, H. J., and C. E. Biro. 1963. Isolation and description of the fourth component of human complement. *J. Exptl. Med.* **118**:447.
10. Polley, M. J., and H. J. Müller-Eberhard. 1967. Enhancement of the hemolytic activity of the second component of human complement by oxidation. *J. Exptl. Med.* **126**:1013.
11. Cooper, N. R., and H. J. Müller-Eberhard. 1968. A comparison of methods for the molecular quantitation of the fourth component of human complement. *Immunochemistry.* **5**:155.

12. Davis, B. J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. *Ann. N. Y. Acad. Sci.* **121**:404.
13. McConahey, P. J., and F. J. Dixon. 1966. A method of trace iodination of proteins for immunologic studies. *Intern. Arch. Allergy Appl. Immunol.* **29**:185.
14. Goudie, R. B., C. H. W. Horne, and P. C. Wilkinson. 1966. A simple method for producing antibody specific to a single selected diffusible antigen. *Lancet.* **ii**:1224.
15. Borsos, T., H. J. Rapp, and M. M. Mayer. 1961. Studies on the second component of complement. I. The reaction between EAC'1,4 and C'2: Evidence on the single site mechanism of immune hemolysis and determination of C'2 on a molecular basis. *J. Immunol.* **87**:310.
16. Mayer, M. M. 1961. Complement and complement fixation. In *Experimental Immunochemistry*. E. A. Kabat and M. M. Mayer, editors. Charles C. Thomas, Springfield, Ill. 133.
17. Andrews, P. 1965. The gel filtration behavior of proteins related to their molecular weights over a wide range. *Biochem. J.* **96**:595.
18. Wyman, J., Jr., and E. N. Ingalls. 1943. A nomographic representation of certain properties of the proteins. *J. Biol. Chem.* **147**:297.
19. Müller-Eberhard, H. J., and I. H. Lepow. 1965. C'1 esterase effect on activity and physicochemical properties of the fourth component of complement. *J. Exptl. Med.* **121**:819.
20. Lepow, I. H., E. W. Todd, R. D. Smink, Jr., and J. Pensky. 1965. Purification of the second component (C'2) of human complement. *Federation Proc.* **24**:446.
21. Polley, M. J., and H. J. Müller-Eberhard. 1965. Isolation of the second component of human complement and the effect of iodoacetamide on its activity. *Federation Proc.* **24**:446.
22. Borsos, T., H. J. Rapp, and C. T. Cook. 1961. Studies on the second component of complement. III. Separation of the second component from guinea pig serum by chromatography on cellulose derivatives. *J. Immunol.* **87**:330.
23. Mayer, M. M., H. S. Shin, and J. A. Miller. 1967. Fragmentation of guinea pig complement components C'2 and C'3c. In *Protides of the Biological Fluids*, Proceedings of the 15th Colloquium. H. Peeters, editor. Elsevier Publishing Co., Amsterdam. 411.
24. Cooper, N. R. 1968. Molecular measurement of the second component (C'2) of human complement (C'). *Federation Proc.* **27**:314.
25. Mayer, M. M., and J. A. Miller. 1965. Inhibition of guinea pig C'2 by rabbit antibody. Quantitative measurement of inhibition, discrimination between immune inhibition and complement fixation, specificity of inhibition and demonstration of uptake of C'2 by EAC'1a,4. *Immunochemistry.* **2**:71.
26. Sitomer, G., R. M. Stroud, and M. M. Mayer. 1966. Reversible adsorption of C'2 by EAC'4: Role of Mg^{2+} , enumeration of competent SAC'4, two-step nature of C'2a fixation and estimation of its efficiency. *Immunochemistry.* **3**:57.
27. Stroud, R. M., M. M. Mayer, J. A. Miller, and A. T. McKenzie. 1966. C'2a^d, an inactive derivative of C'2 released during decay of EAC'4,2a. *Immunochemistry.* **3**:163.