Third Component of Human Complement: Purification from Plasma and Physicochemical Characterization†

B. F. Tack and J. W. Prahl*

ABSTRACT: The third component of complement has been purified from fresh human plasma employing an initial fractionation with poly(ethylene glycol) followed by sequential depletion of plasminogen by affinity adsorbents, chromatography on diethylaminoethylcellulose, gel filtration on agarose, and batch adsorption/desorption on hydroxylapatite. Final recoveries of C3 were 33% of the initial protein, as quantitated by radial immunodiffusion, and 31% of the initial hemolytic activity. Apparent homogeneity is indicated by immunological criteria and by polyacrylamide gel electrophoresis. A partial specific volume of 0.736 ± 0.003 ml gm^{-1} was determined for C3 by the mechanical oscillator technique. "Low speed" sedimentation equilibrium yielded an apparent weight average molecular weight for the protein of 187 650 ± 5650. Based upon this molecular weight, a molar extinction coefficient of 1.82×10^5 l. $mole^{-1}$ cm^{-1} at 280 nm was calculated from boundary-spreading experiments in the ultracentrifuge and an assumed refractive index increment. Amino acid analyses revealed no unusual or distinctive characteristics. Automated Edman degradation revealed a double N-terminal sequence, Ser-Val,Pro-Glx,Met-Leu,Tyr-Thr,Ser-Glx,Ile-Lys,Gly-Arg,Thr-Met,Pro-Asx, in agreement with the two chain structure observed on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, and revealing both chains are available to degradation. Serine is postulated as the initiating sequence in both chains based upon high recoveries of dinitrophenylserine upon hydrolysis of dinitrophenylated C3, and our inability to identify any other dinitrophenyl or phenylthiohydantoin derivatives in this position. Alanine is the ultimate carboxyl-terminal amino acid of at least one of the chains, as indicated by the action of carboxypeptidases on C3 in the presence of sodium dodecyl sulfate.

The complement system of vertebrates functions as a humoral biological effector following antigen-antibody interaction. Complement shares many similarities with the complex of coagulation proteins, which may reflect the similar evolutionary pressures operative on both systems. Both the complement and the coagulation systems are characterized by their multimolecular complexity, activation being achieved in a sequential or "cascade" process, with the presence of inhibitors and inactivators to achieve control and fine tuning of the activation process, and, finally, the existence of alternative pathways of activation and feedback control (Müller-Eberhard, 1975; Prahl, 1976).

The third component of complement, C3, plays a central role as the protein at which the classical and alternate pathways of activation converge. C3 may interact with C3 convertase, $\overline{C4,2}$, via classical activation to give rise to C5 convertase, $\overline{C4,2,3}$ (Müller-Eberhard et al., 1967), or with the proteins of the alternate pathway giving rise to an alternate pathway C3 convertase, $\overline{C3b,Bb}$ (Sandberg et al., 1970; Götze and Müller-Eberhard, 1971). The activation of C3 is accompanied by the appearance of anaphylactic activity (DaSilva et al., 1967) which correlates with the formation of a small activation peptide of C3, i.e., C3a (Bokisch et al., 1969). The remaining activation fragment of C3, i.e., C3b, interacts with membrane in an unknown manner, conferring the topographical constraints observed in the lytic activity of complement (Müller-Eberhard et al., 1966).

The existence of C3b receptors on monocytes, macrophages, polymorphonuclear leukocytes, and B cells has emphasized the role of complement in opsonization, immune adherence, and the humoral immune response (Lay and Nussenzweig, 1968; Huber et al., 1968; Miller and Nussenzweig, 1974; Bianco et al., 1975).

In view of the significant biological role of C3 in complement-mediated phenomena and its relative abundance in blood (80–130 mg/100 ml of serum), this protein was chosen to commence structural studies of the complement system. Isolation of human C3 from serum was first reported by Müller-Eberhard et al. (1960) with later revisions (Nilsson and Müller-Eberhard, 1965), but the recovery of C3 was usually less than 5% of theoretical. A recent report by Molenaar et al. (1973), also utilizing serum, improved the yield to 15% but required the use of affinity adsorbents made of antisera. The present report describes an improved preparative procedure of human C3 starting from plasma with recoveries in excess of 30%. The physicochemical characteristics of C3 prepared in the manner to be described are presented.

Experimental Procedures

Materials. Platelet-poor plasma of freshly drawn human blood was obtained from the Washington Regional Blood Center of the American National Red Cross. Sepharose 4B and 6B were obtained from Pharmacia Chemicals; EDTA,[1] PMSF, L-lysine, EACA, Tris, DFP, and proteins to be used as molecular weight markers in polyacrylamide gel electrophoresis were purchased from Sigma. Microgranular DEAE-cellulose (Whatman DE-52) was a product of Reeve

† From the Immunochemistry Section of the American National Red Cross Blood Research Laboratory, Bethesda, Maryland 20014. *Received February 11, 1976.* Publication No. 340. This work was supported in part by United States Public Health Service Research Grant AI 12098.

[1] Abbreviations used are: EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; EACA, ϵ-amino-*n*-caproic acid; DFP, diisopropyl fluorophosphate; DNP, dinitrophenyl; DEAE, diethylaminoethyl; PEG, poly(ethylene glycol); Temed, *N,N,N′,N′*-tetramethylethylenediamine; DTT, dithiothreitol; PBS, 10 mM sodium phosphate buffer (pH 7.4) containing 150 mM NaCl; GVB^{2+}, 4.94 mM sodium Veronal buffer containing 142 mM NaCl, 0.1% gelatin, 0.3 mM $CaCl_2$, and 1.0 mM $MgCl_2$; Pth, phenylthiohydantoin; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

Angel. Hydroxylapatite (Bio-Gel HT), electrophoresis grade acrylamide, *N,N'*-methylenebisacrylamide, Temed, and ammonium persulfate were purchased from Bio-Rad Laboratories; mercaptoethanesulfonic and methanesulfonic acids, and sequenator reagents were from Pierce Chemical Co.; the linear polymer, PEG 4000, was from Union Carbide; Diaflo ultrafiltration membranes were from Amicon. Functionally pure human C3, C2, and $EAC1_{gp},4_{hu}$ cells were obtained from Cordis Laboratories. all other chemicals and reagents used were of the highest grade available.

Antisera. Rabbit antisera to human C3, C4, IgA, IgM, IgG, albumin, transferin, ceruloplasmin, plasminogen, fibrinogen, and α_2-macroglobulin were purchased from Behringwerke. Goat anti-human C5 was obtained from Meloy Laboratories. A guinea pig anti-human C3/C5 was raised using post-Sepharose C3/C5 as antigen. Equal volumes of protein (10 mg/ml) and complete Freunds adjuvant were emulsified and a total of 1 ml/animal was injected subcutaneously at multiple sites including the footpads. Two weeks later, the animals were boosted with 1.0 ml of antigen (2.5 mg/ml). Bleeding was commenced 1 month after initial inoculation, and repeated every 3–4 days. Pools were made on the basis of the specificity of the antisera obtained.

Hemolytic Assays. The hemolytic activity of C3 was followed during purification using $EAC1_{gp},4_{hu}$ cells and an R3 reagent depleted of C3 activity and, to a lesser extent, of C4 and C5 activities. The R3 reagent was prepared according to Dalmasso and Müller-Eberhard (1964) by incubating a volume of fresh human serum with an equal volume of saturated KBr for 18 h at 4 °C. The KBr-treated serum was dialyzed against PBS, aliquoted, and stored at −70 °C. Qualitative assays containing 0.4 ml of EAC1,4 cells (1×10^8 cells/ml), 0.05 ml of 1:5 R3 reagent, 0.02–0.05 ml of sample, and 0.50–0.53 ml of GVB^{2+} were incubated for 10 min at 37 °C. The recovery of C3 hemolytic activity was quantitatively determined using 2×10^7 EAC1,4 cells, and incubation at 37 °C for 1 h, where the hemolytic unit, CH_{50}, was the reciprocal of the dilution of C3 that would lyse 1×10^7 cells (Mayer, 1961).

The hemolytic activity of C5 was followed using $EAC1_{gp},4_{hu}$ cells and an R5 reagent depleted of C3, C4, C2, and C5. The R5 reagent was prepared according to Cooper and Müller-Eberhard (1970) by incubating a volume of human serum with an equal volume of 1 M KSCN for 18 h at 4 °C and further incubation for 45 min at 37 °C after being made 1.5×10^{-2} M with hydrazine. The treated serum was then dialyzed against PBS, aliquoted, and stored at −70 °C. Here the qualitative C5 assay contained 0.40 ml of $EAC1_{gp},4_{hu}$ (1×10^8 cells/ml), 0.05 ml of 1:5 diluted R5 reagent, 0.01 ml of C2 (1000 CH_{50} units/ml), 0.05 ml of C3 (1000 CH_{50} units/ml), 0.01–0.05 ml of sample, and 0.44–0.48 ml of GVB^{2+}. Quantitative determination of C5 hemolytic activity was performed with 2×10^7 cells as described in the case of C3.

Immunological Techniques. Total C3 protein, i.e., native C3 and C3b, was quantitated by radial immunodiffusion according to Mancini et al. (1965). The C3 immunodiffusion plates (Behringwerke) were developed following the manufacturers instructions. Immunoelectrophoresis in 1% agarose was performed according to Scheidegger (1955). Ouchterlony analyses were carried out on plates purchased from Hyland Laboratories.

Polyacrylamide Gel Electrophoresis. C3 preparations were examined by polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate according to the procedures of Weber and Osborn (1969). The monomer acrylamide concentration of the gels was 5.87% with an acrylamide/bisacrylamide ratio of 87:1. Samples were prepared by dialysis against 10 mM sodium phosphate (pH 7.0) and 1% sodium dodecyl sulfate overnight at room temperature. Prior to electrophoresis, the samples were reduced with 1% 2-mercaptoethanol for 2 h at 37 °C. In those instances in which the samples were initially in guanidinium chloride, they were first dialyzed against 10 mM sodium phosphate (pH 7.0) containing 6 M freshly deionized urea, and finally the sodium dodecyl sulfate buffer. *Escherichia coli* β-galactosidase, rabbit muscle phosphorylase, *Aspergillus niger* glucose oxidase, beef liver catalase, and pig heart fumarase were used as molecular weight markers, assuming values of 130 000, 100 000, 75 000, 60 000, and 49 000, respectively.

Reduction and Alkylations. Salt-free samples were dissolved in 0.2 M Tris-HCl buffer (pH 8.2) containing 6 M guanidinium chloride and 5 mM EDTA, to 10–20 mg of protein/ml. They were made 20 mM with respect to DTT and incubated for 2–3 h at 37 °C under nitrogen. Alkylation was accomplished by the addition of iodoacetamide to 42 mM. When ethyleneimine was used as the alkylating agent, the buffer contained 1.5 M Tris-HCl (pH 9.0), 6 M guanidinium chloride, and 5 mM EDTA. A fivefold molar excess of ethyleneimine (to 240 mM) was added relative to the thiol concentration.

Preparation of a Biospecific Adsorbent for Plasminogen. L-lysine was coupled to CNBr-activated Sepharose 4B following the procedure described by Deutsch and Mertz (1970). Hydrolysis of a washed and dried sample of the adsorbent released 71 μmol of lysine/ml of Sepharose, assuming 52 mg of dried gel equivalent to 1 ml of settled hydrated gel. Prepared in this manner, the adsorbent was found to bind small amounts of IgG, C3, and C4 which could be eluted with 0.3 M potassium phosphate buffer (pH 7.4). Plasminogen was eluted with 0.2 M EACA in 0.3 M potassium phosphate (pH 7.4), and the gel was washed quickly with 0.01 M NaOH followed by 0.01 M H_3PO_4. After copious water washes, the gel was stored at 4 °C in 0.02% sodium azide.

Molar Extinction Coefficient of C3. A synthetic boundary determination of C3 concentration was carried out in the An-H Ti rotor in a 12-mm double-sector cell equipped with interference window holders, sapphire windows, and a double-sector capillary synthetic-boundary centerpiece. The C3 preparation was dialyzed against 10 mM Tris-HCl buffer (pH 8.0), 0.2 mM in NaCl, and the absorbance at 280 nm was determined just prior to the synthetic boundary run. At a rotor speed of 8000 rpm, when the menisci had equalized and the boundary had diffused sufficiently to give clear fringes, a photograph was taken. Additional photographs were recorded for 60 min at 15-min intervals. The concentration in the plateau region was determined by estimating the total fringe shift across the boundary, assuming a shift of one fringe to correspond to a protein concentration of 0.25 mg of protein/ml in the 12-mm cell (Chervenka, 1969). An extinction coefficient $\epsilon_{1cm}^{1\%}$ at 280 nm of 9.7 was determined, and a molar extinction coefficient of 1.82×10^5 l. mol^{-1} cm^{-1} calculated based on a molecular weight of 187 650.

Sedimentation Equilibrium Analysis of C3. The conventional or "low speed" sedimentation equilibrium method was used to determine the molecular weight of C3 (Teller, 1973). The C3 preparation was dialyzed against 10 mM Tris-HCl buffer (pH 8.0) containing 0.2 M NaCl, and diluted with dialysate to give solutions of 0.4, 0.2, and 0.1 mg/ml of protein. Equilibrium runs were carried out in the six hole An-G Ti rotor in a Beckman Model E analytical ultracentrifuge equipped

with a photoelectric scanner, multiplexer, and electronic speed control. Samples were run in 12-mm double-sector cells equipped with absorption window holders and sapphire windows. A single run at the three protein concentrations was carried out at a rotor speed of 6000 rpm. Once equilibrium had been established and a final scan of each cell was recorded, the rotor was accelerated to 48 000 rpm to clear the top half of the liquid column, decelerated to 6000 rpm, and each cell was rescanned to establish the position of zero absorbance. The protein concentration (C) was expressed in units of absorbance at 280 nm and reduced molecular weights ($M(1 - \bar{v}\rho)$) calculated from the equation

$$M(1 - \bar{v}\rho) = \frac{2RT}{\omega^2} \cdot \frac{\mathrm{d}(\ln C)}{\mathrm{d}r^2}$$

where $\mathrm{d}(\ln C)/\mathrm{d}r^2$ was derived from the slope of a ln C vs. r^2 plot. The apparent weight average molecular weight of C3 was calculated from the reduced molecular weight using an experimentally derived value for the partial specific volume ($\bar{v}$) of the protein.

Partial Specific Volume ($\bar{v}$) of C3. The partial specific volumes of bovine serum albumin and C3 were determined from density measurements with a precision density meter DMA 02C (Anton Paar, Gratz) as described by Lee and Timasheff (1974). Samples were dialyzed for a minimum of 48 h against 10 mM Tris-HCl buffer (pH 8.0) containing 0.2 M NaCl, and the absorbances at 280 nm were determined just prior to density measurements. The density difference between two samples ($d_1 - d_2$) is given by the equation

$$d_1 - d_2 = \frac{1}{A}(T^2_1 - T^2_2)$$

where T is the time required for a preset number of periods and A an instrument constant calculated using dry air and distilled water as standards for 2×10^4 periods. The temperature of the cell was maintained at 21.0 °C and the densities of air and water at this temperature were found in the critical tables (Hodgman, 1961). The apparent partial specific volume ($\bar{v}_{app}$) of the solute is found by solving

$$\bar{v}_{app} = \frac{1}{d_0}\left(1 - \frac{(d - d_0)}{c}\right)$$

where d_0 is the density of the solvent, d the density of the protein solution, and c the concentration of protein (solute) in g/ml.

Amino Acid Analysis. Amino acid analyses were performed on a JEOL 6-AH amino acid analyzer coupled to an Autolab System AA computing integrator. Samples were hydrolyzed for 24, 48, and 72 h with two times glass-distilled constant-boiling 6 N HCl at 110 °C in sealed tubes after evacuation to less than 5×10^{-3} Torrs. Tryptophan was determined by hydrolysis with 3 N mercaptoethanesulfonic acid (Penke et al., 1974). Serine and threonine values were corrected for destruction by extrapolation to zero time. Maximal values were taken for all other amino acids.

Sequence Analysis. Automated sequence analysis of reduced and alkylated C3 was carried out on a JEOL Model 47K sequence analyzer using a modified double-cleavage program with 0.25 M Quadral. The protein, 100–150 nmol, was loaded in the cup in 0.1% sodium dodecyl sulfate in an effort to improve solubility in the coupling steps. Known amounts of Pth-norleucine were placed in the fraction collector tubes or in heptafluorobutyric acid as an internal standard. The thiazolinones were converted to Pth amino acids and identified on a Packard gas chromatograph on SP400 (Pisano and Bronzert, 1969) and by back hydrolysis with HI (Smithies et al., 1971). For identification of particular residues, high-pressure liquid chromatography (Zimmerman et al., 1973), thin-layer chromatography (Summers et al., 1973), and the arginine stain of Itano and Robinson (1972) were employed.

Miscellaneous Methodology. Protein analyses were performed using a modification of the Lowry method (Hartree, 1972). Carboxypeptidase A and/or B digestions were performed in either 0.1 M sodium phosphate buffer (pH 7.8), containing 0.1% sodium dodecyl sulfate, at a substrate to enzyme molar ratio of 16:1 to 20:1 or in 0.2 M bicarbonate buffer (pH 8.9) containing 0.1% sodium dodecyl sulfate. The digestion was stopped by acidification with 6 N HCl to pH 2.2 and the amino acids released were quantitated on the amino acid analyzer in the presence of internal standard.

Purification Procedure. All operations were performed at 4 °C unless otherwise stated.

Step 1: Fractionation of Plasma with PEG. Twelve units of freshly drawn human plasma were individually chilled in an ice bath and made 0.5 mM in PMSF by the addition of an anhydrous 2-propanol solution of PMSF (20 mg/ml). A volume equivalent to one-half that of each plasma volume of 15% (w/v) PEG 4000 in a buffer containing 100 mM sodium phosphate (pH 7.4), 150 mM NaCl, 15 mM EDTA, and 0.5 mM PMSF, was slowly added with stirring to each unit, resulting in a final PEG concentration of 5%. After standing for 30 min, the precipitate was removed by centrifugation at 5000g for 15 min and the individual supernatants were pooled. The PEG concentration was raised to 12% by the addition of 26% (w/v) PEG in the same buffer as above, slowly with stirring. After another 30 min, the precipitate was collected by centrifugation at 7000g for 20 min and the supernatant was discarded. The 5–12% PEG precipitate was dissolved in 400 ml of the same buffer by gentle agitation with glass stirring rods over a period of 45 min.

Step 2: Plasminogen Depletion of the 5–12% PEG Fraction. A volume of 350–400 ml of settled L-lysine–Sepharose 4B was equilibrated with the buffer described in step 1. The biospecific adsorbent was taken to dryness by suction on a fritted glass filter of medium porosity and suspended in the resolubilized 5–12% PEG fraction with gentle stirring. After several hours, the unadsorbed protein was removed by gentle vacuum filtration on the glass filter and the adsorbent was washed successively with two 100-ml aliquots of the resolubilizing buffer. All the filtrates were pooled for chromatography on DEAE-cellulose. An alternate method for plasminogen depletion employed was to pack the equilibrated L-lysine–Sepharose biospecific adsorbent in a column 5.0 cm in diameter and to pump the resolubilized 5–12% PEG fraction and subsequent washes through at the rate of 125–150 ml/h.

Step 3: DEAE-Cellulose Chromatography. The plasminogen depleted 5–12% PEG fraction was further diluted to a specific conductance of 3.0 mmho/cm (4 °C) with cold 5 mM EDTA. The pH was adjusted to 7.0 with cold glacial acetic acid or 1 N NaOH, if necessary, and made 50 mM in EACA by the addition of the solid. The sample was applied to a 5.0 × 160 cm column of DEAE-cellulose equilibrated with 25 mM potassium phosphate buffer (pH 7.0) containing 5 mM EDTA at a flow rate of 140 ml/h. Once the sample was loaded, the column was washed with 3.2 l. of 25 mM potassium phosphate buffer (pH 7.0), containing 5 mM EDTA and 50 mM EACA, and then developed with a linear NaCl gradient (total volume of 8 l.) in the same buffer to a limiting concentration of 300 mM NaCl. The flow rate was maintained at 125 ml/h, 18-ml fractions were collected, and the absorbance at 280 nm was

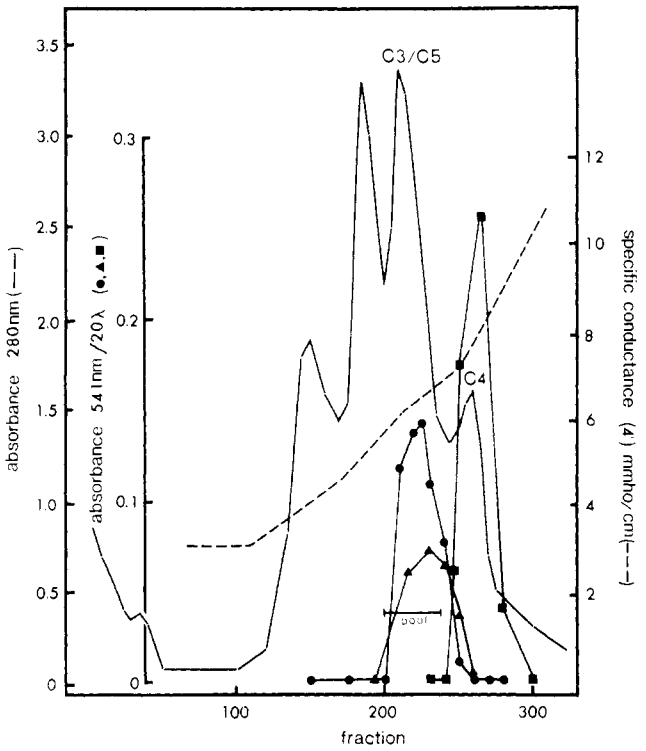

FIGURE 1: Ion-exchange chromatography of 12 units of the plasminogen-depleted 5–12% PEG fraction of fresh human plasma on a 5 × 160 cm column of DEAE-cellulose. The column had been equilibrated with 25 mM potassium phosphate buffer (pH 7.0) containing 5 mM EDTA. The sample, at pH 7.0 and a specific conductance of 3.0 mmho/cm (4 °C), was made 50 mM with EACA and loaded on the column. After a wash with 25 mM potassium phosphate buffer (pH 7.0) containing 5 mM EDTA and 50 mM EACA, a linear gradient of NaCl to 300 mM (8-l. total) was developed. Absorbance at 280 nm (—), specific conductivity (- - -), and hemolytic activities of C3 (●—●), C5 (▲—▲), and C4 (■—■) are shown. C3/C5 containing fractions were pooled as indicated.

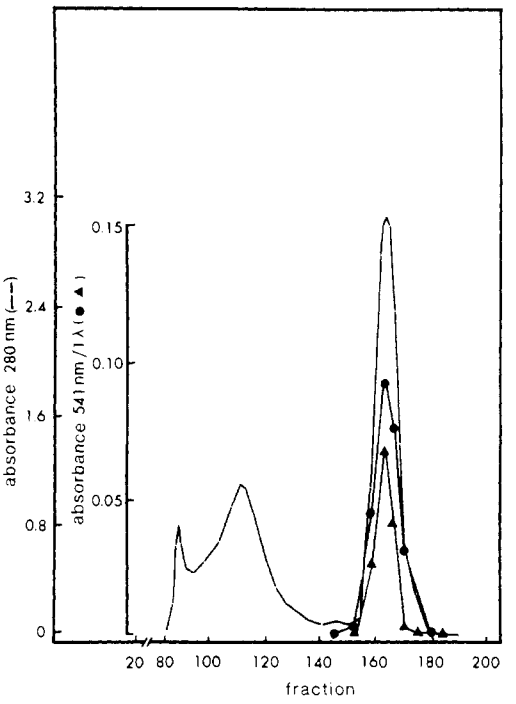

FIGURE 2: Gel filtration of the post-DEAE C3/C5 pool on a 5.0 × 60 cm column of Sepharose 6B equilibrated and developed with 100 mM potassium phosphate buffer (pH 7.4) containing 150 mM NaCl, 5 mM EDTA, and 50 mM EACA. Absorbance at 280 nm (—) and hemolytic activities of C3 (●—●) and C5 (▲—▲) are shown.

monitored. The elution profile revealed resolution of four major peaks (Figure 1). The third peak, fractions 200–250, contained C3 and C5 hemolytic activities, eluting at a specific conductance of 6.5 mmho/cm (4 °C). Ceruloplasmin was detected by its blue color at the trailing edge of this peak. C4 hemolytic activity was found in the fourth peak, fractions 250–280, eluting at a specific conductance of 7.5 mmho/cm (4 °C).

The fractions containing C3/C5 were pooled and concentrated by reprecipitation with PEG by the addition of sufficient solid to give a final PEG concentration of 16%. The precipitate was collected by centrifugation and dissolved in 60 ml of 100 mM potassium phosphate buffer (pH 7.4), containing 150 mM NaCl, 5 mM EDTA, and 50 mM EACA, and then made 0.5 mM with PMSF.

Step 4: Gel Filtration on Sepharose 6B. The post-DEAE C3/C5 pool was subjected to gel filtration in two equal aliquots on a 5.0 × 160 cm column of Sepharose 6B equilibrated with 100 mM potassium phosphate buffer (pH 7.4) containing 150 mM NaCl, 5 mM EDTA, and 50 mM EACA. A thirty-milliliter sample was loaded at a hydrostatic differential of some 40 cm and the column was then pumped at a flow rate of 90 ml/h in the downward direction. Twelve-milliliter fractions were collected and the absorbance at 280 nm was monitored. The protein was resolved into two major peaks (Figure 2) which eluted at 42 and 62% of the column bed volume, respectively. The fractions containing C3 and C5 hemolytic activity, 154–169, were pooled, made 0.5 mM in PMSF, and concentrated to approximately 7 mg/ml by ultrafiltration on an Amicon XM 50 membrane.

Step 5: Hydroxylapatite Chromatography. The post-Sepharose C3/C5 pool was dialyzed against 25 mM potassium phosphate buffer (pH 7.4) containing 100 mM KCl and 50 mM EACA, and then diluted to 5 mg/ml with this buffer. The sample was processed in two equal aliquots on a 5.0 × 40 cm column of hydroxylapatite equilibrated with the sample buffer. After application of the sample, the column was washed with 1.2 l. of the sample buffer at a flow rate of 125 ml/h, collecting 16-ml fractions in tubes containing 0.8 ml of 100 mM EDTA, pH 7.4. The starting buffer was followed by 1.2 l. of 25 mM potassium phosphate buffer (pH 7.4) containing 2 M KCl and 50 mM EACA, backwashed with 1.2 l. of starting buffer, and finally stripped with 125 mM potassium phosphate buffer (pH 7.4) containing 100 mM KCl and 50 mM EACA. A wash with 0.4 M potassium phosphate buffer (pH 7.4) before equilibration with starting buffer was performed, and the second half of the C3/C5 pool was processed in an identical fashion.

Only C5 hemolytic activity was identified in the 2 M KCl wash of the hydroxylapatite column (Figure 3; fractions 50–80), the elution of C3 requiring the presence of high phosphate (Figure 3; fractions 180–210). The appropriate C3 and C5 fractions were pooled, concentrated by ultrafiltration on an Amicon XM 50 membrane, and stored at −70 °C. C3 purified through the hydroxylapatite step was used for all subsequent studies.

Results and Discussion

Comments on the Purification Scheme. Platelet-poor plasma of freshly drawn human blood served as the starting material of the purification scheme described here. The use of plasma was predicated by (1) its availability as a by-product in the preparation of packed erythrocytes and platelets, as-

TABLE I: Summary of C3 Purification.

Step	Total C3[a] (mg)	Yield (%)	Total C3[b] (CH_{50} Units)	Yield (%)
1. Plasma	1692	100	23.8×10^6	100
2. 5–12% PEG precipitate	1368	81	15.5×10^6	65
3. Post-DE52 C3/C5 Pool	1245	74	17.6×10^6	74
4. Post-Sepharose 6B C3/C5 Pool	910	54	13.3×10^6	56
5. Post-Bio-Gel-HT C3 Pool	553	33	7.3×10^6	31

[a] Determined by an immunoprecipitant technique (Mancini). [b] Determined by molecular titration using a $EAC1_{gp}4_{hu}$ cell intermediate and KBr-treated human serum for a C2 and C5–C9 source.

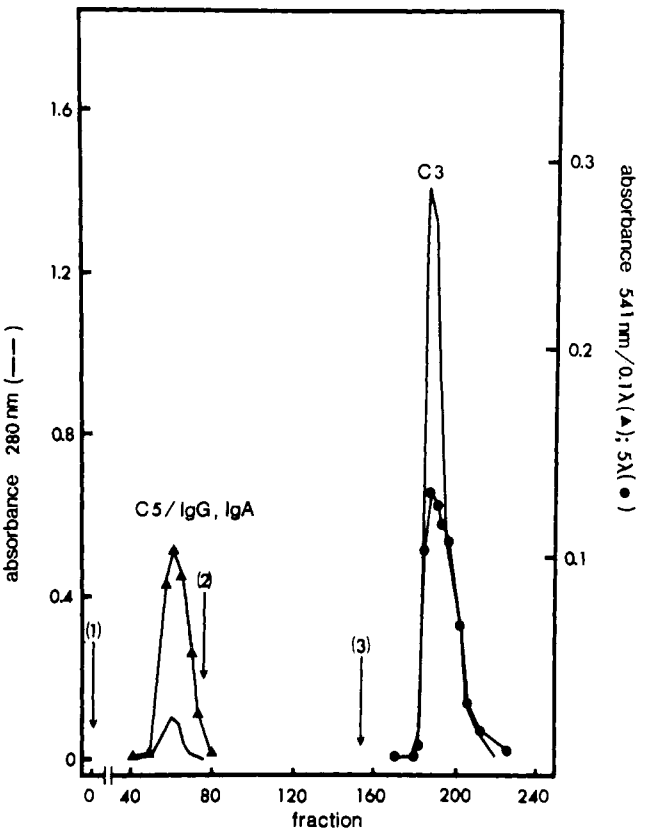

FIGURE 3: Hydroxylapatite chromatography of the post-Sepharose 6B pool. A 5 × 40 cm column of hydroxylapatite was equilibrated with 25 mM potassium phosphate buffer (pH 7.4) containing 100 mM KCl and 50 mM EACA. A sample of 500 mg of protein was applied in the same buffer at a concentration of 5 mg/ml. After a wash with the starting buffer, the first peak containing C5/IgG, IgA was eluted with 25 mM potassium phosphate buffer (pH 7.4) containing 2 M KCl and 50 mM EACA (arrow 1). Following a second wash with the starting buffer (arrow 2), the C3 was eluted with 125 mM potassium phosphate buffer (pH 7.4) containing 100 mM KCl and 50 mM EACA (arrow 3). Absorbance at 280 nm (—), and hemolytic activity of C3 (●—●) and C5 (▲—▲) identified are shown.

suring maximal use of natural resources, (2) our desire to avoid activation of the clotting factors, and (3) the ability to remove fibrinogen with the linear polymer, polyethylene glycol (PEG) 4000. PEG has been shown to be an effective fractionating agent for plasma proteins (Polson et al., 1964) and capable of removing the bulk of fibrinogen at 5% (w/v) concentration. Undissociated C1 and C1q are also found in this fraction, accounting for the reported loss of complement activity by Dalmasso and Müller-Eberhard (1964), as there was no evidence of activation of C3 or C4 in our hands utilizing PEG as a fractionating agent. The 5–12% PEG fraction was found to contain 80% of the initial plasma C3, but only 28% of the initial plasma protein (Table I). The concentration of an inhibitor in this fraction was presumed to be responsible for the decreased C3 hemolytic activity observed, in view of the increased activity observed in subsequent steps of the purification. Whereas C3, C5, and C4 were effectively concentrated in the 5–12% PEG precipitate, albumin and transferrin remain soluble in the 12% PEG supernatant, making chromatography on DEAE-cellulose reasonable for starting volumes as large as 12–20 units of plasma. Fresh frozen plasma was found to be a suitable alternative starting material for the purification of C3 and C5.

The 5–12% PEG fraction was shown immunologically to contain considerable plasminogen/plasmin. Endogenous plasmin or plasmin formed by the autocatalytic activation of plasminogen are capable of activating C3 (Bokisch et al., 1969). Despite efforts to control this potential hazard by the incorporation of EACA and the serine protease inhibitors PMSF and DFP in the purification scheme, sporadic activation of C3 was best controlled by plasminogen depletion utilizing the biospecific adsorbent, L-lysine-agarose. A relatively high ionic strength was maintained during the depletion step, as the adsorbent prepared by the method described demonstrated weak ion-exchange properties (Nishikawa and Bailon, 1975). The conductivity of the depleted PEG fraction was then dropped preparative to the chromatography on DEAE-cellulose.

The post-DEAE C3/C5 pool contained 74% of the initial plasma C3 hemolytic and antigenic activities (Table I). In addition to C3 and C5, IgG, IgA, IgM, and α_2-macroglobulin were identified in the pool. The IgM and α_2-macroglobulin, representing about 29% of the total absorbance at 280 nm, were resolved from the C3/C5 by gel filtration on Sepharose 6B where they eluted at 42% of the total column bed volume. The post-Sepharose C3/C5 pool accounted for 54% of the initial plasma C3 antigenic activity and 56% of the initial C3 hemolytic activity (Table I). Quantitation by radial immunodiffusion revealed the pool was composed of 85% C3, 9.5% C5, 4.2% IgG, and <0.4% IgA.

Resolution of C3 and C5 has been routinely effected on hydroxylapatite utilizing increasing phosphate concentration (Nilsson and Müller-Eberhard, 1965; Nilsson et al., 1972). The method employed in the purification described here takes advantage of the selective desorption of C5, IgG, and IgA by high chloride concentrations (Hjertén, 1959). Although the potassium salt has been used for reasons of solubility, there is some evidence that the C5 so eluted undergoes partial denaturation. Preliminary experiments suggest this problem may be circumvented by the use of sodium chloride. The post-hydroxylapatite C3 pool contained 33% of the initial plasma C3 protein and 31% of the initial plasma hemolytic activity (Table I). The specific hemolytic activity of this C3 was determined to be 12 195 CH_{50} units/mg of protein.

Immunochemical Analyses. Antiserum made in the guinea pig using the post-Sepharose C3/C5 pool as antigen demonstrated multivalency, as anticipated. Immunoelectrophoresis of the antigen revealed three precipitin lines with the antiserum, a major anti-C3 and a minor anti-C5 line of β mobility, and a weak anti-IgG/IgA line extending from the well into the β region (Figure 4a, upper well). The post-hydroxylapatite C3, on the other hand, gave only a single precipitin line with this antiserum (Figure 4a, lower well), as well as a single β-mi-

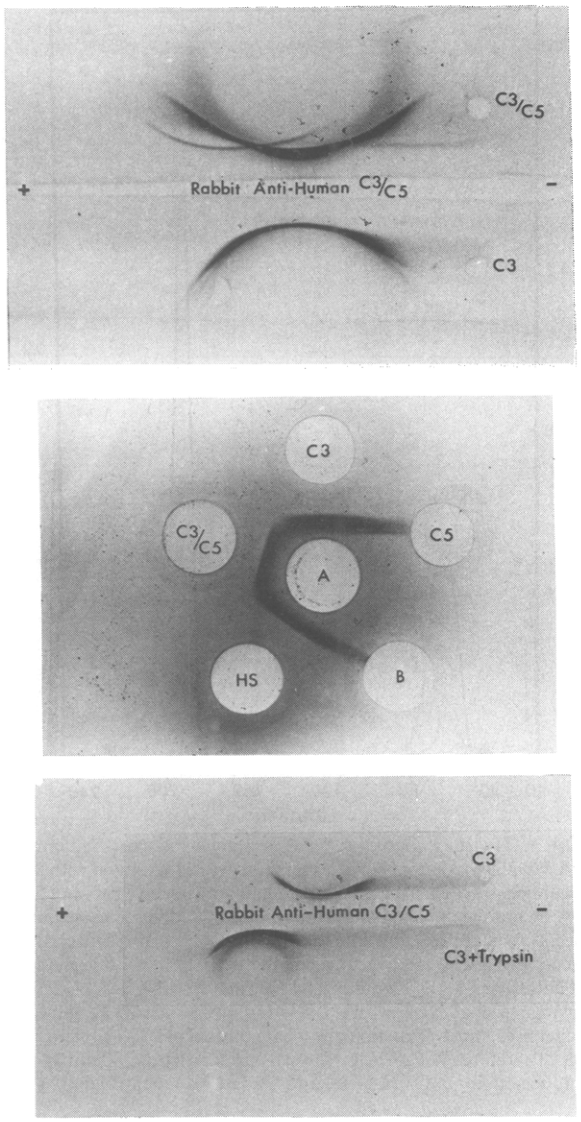

FIGURE 4: (a, top) Immunoelectrophoretic pattern of the post-Sepharose C3/C5 pool (upper well) and post-hydroxylapatite C3 (lower well) developed with guinea pig anti-human post-Sepharose C3/C5 in the trough; (b, middle) diffusion Ouchterlony analysis of post-Sepharose C3/C5, post-hydroxylapatite C3, post-hydroxylapatite C5, and human serum against rabbit anti-human post-hydroxylapatite C3 prepared in this laboratory; (c, bottom) Immunoelectrophoretic pattern of post-hydroxylapatite C3 (upper well) and the same material after trypsinization developed with guinea pig anti-human post-Sepharose C3/C5 in the trough.

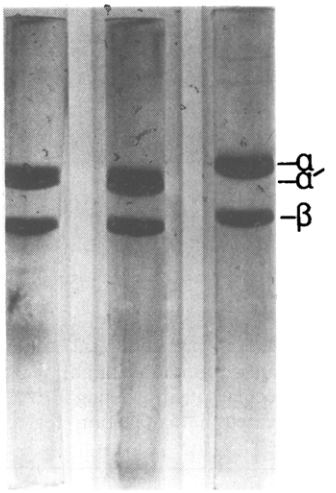

FIGURE 5: The subunit structure of post-hydroxylapatite C3 as revealed by polyacrylamide gel electrophoresis in 0.1% sodium dodecyl sulfate according to Weber and Osborn (1969). From right to left the gels are: (a) reduced post-hydroxylapatite C3; (b) trypsinized and reduced post-hydroxylapatite C3; (c) a mixture of 50% (a) and 50% (b).

grating line with rabbit anti-whole human serum. Rabbit antiserum prepared in this laboratory against the post-hydroxylapatite C3 was unreactive with the post-hydroxylapatite C5 pool and gave a single precipitin line with stabilized human serum, the post-Sepharose C3/C5 pool, and the post-hydroxylapatite C3 (Figure 4b) upon double diffusion in gel. Goat anti-human C5 was unreactive with the post-hydroxylapatite C3 in gel diffusion at an antigen concentration of 4.5 mg of protein/ml, although rabbit anti-human IgG revealed trace contamination. This was quantitated at less than 0.2% (w/w) IgG impurity by radial immunodiffusion.

Native C3 exhibits a characteristic anodal shift in electrophoretic mobility on conversion to C3b with the EAC$\overline{1,4,2}$ cell intermediate (Müller-Eberhard et al., 1966) with C$\overline{4,2}$, trypsin, or plasmin (Bokisch et al., 1969), and with 0.5 M hydroxylamine (Budzko and Müller-Eberhard, 1969). Post-hydroxylapatite C3 similarly demonstrated this anodal shift after treatment for 1 min with 1% trypsin (w/w) at pH 7.4 (Figure 4c, upper well) relative to the untrypsinized control (Figure 4c, lower well).

Polyacrylamide Gel Electrophoresis Analyses. Post-hydroxylapatite C3 consistently revealed two polypeptide chains on sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis after denaturation and reduction (Figure 5, gel 3), in agreement with the observations reported by others (Nilsson and Mapes, 1973; Bokisch et al., 1975; Molenaar et al., 1974). The polypeptide chains, α and β, were assigned respective molecular weights of 115 000 ± 11 500 and 75 000 ± 7000 by comparison of their electrophoretic mobilities with those of proteins of known molecular weight. These constituent chains of C3 do not dissociate in the absence of reducing reagent, implying the quaternary structure of the native protein is stabilized by inter-chain disulfide bonds. Activation of C3 has been shown to result in the proteolytic degradation of the α chain, resulting in an α' chain some 8000–9000 daltons smaller than the native chain (Nilsson and Mapes, 1973; Bokisch et al., 1975). The α' chain is clearly discernible from the α chain on sodium dodecyl sulfate–polyacrylamide gel electrophoresis and serves as a further criterion that post-hydroxylapatite C3 contains no activated C3 (Figure 5, gels 1 and 2).

Molecular Weight Determinations by Sedimentation Equilibrium. Weight average molecular weights for post-hydroxylapatite C3 were obtained by the "low speed" sedimentation equilibrium method at three protein concentrations using absorption optics and a split-beam photoelectric scanner. A plot of the ln of the recorded absorbance at 280 nm against the square of the distance from the axis of rotation for each solute concentration was obtained by a linear least-square fit of the data using the NIH M Lab/PDP10 interactive system described by Knott and Shrafer (1972). The plots for the three samples were parallel, implying negligible contribution by self-associative phenomena in the concentration range investigated. Determined slopes and reduced molecular weights at the three concentrations are shown in Table II. Apparent weight average molecular weights were calculated using a $\bar{v}$ of 0.736 ± 0.003 ml g^{-1} determined by the mechanical oscillator technique of Lee and Timasheff (1974). The precision of the method should permit derivation of a $\bar{v}$ value with

TABLE II: Reduced and Calculated Weight Average Molecular Weight for C3 by Conventional Sedimentation Equilibrium.

Run	Slope $(d(\ln C)/dr^2)$	$M(1-\bar{v}\rho)$	Mol Wt	
1	0.412	48 780	188 300	187 650 ± 5650
2	0.417	49 400	190 800	
3	0.402	47 600	183 850	

[a] The partial specific volume ($\bar{v}$) of C3 was calculated to be 0.737 ml g^{-1} from the amino acid composition (Cohn and Edsall, 1943) and 0.736 ± 0.003 ml g^{-1} by density measurements with a precision density meter (DMA-02C; Anton Paar, Gratz). Runs 1-3 correspond to C3 concentrations of 0.4, 0.2, and 0.1 mg/ml, respectively.

0.2-0.5% accuracy. No distinction was made between the "effective" and the apparent specific volumes determined here. Interestingly, a $\bar{v}$ of 0.737 ml g^{-1} was calculated from the amino acid composition of this C3 (Cohn and Edsall, 1943), not taking into consideration the contribution of the 1.5% content of carbohydrate.

The molecular weight of 187 650 ± 5650 obtained for C3 by sedimentation equilibrium analysis (Table II) is in fair agreement with that of 180 000 reported by Bokisch et al. (1969) using the Archibald approach to sedimentation equilibrium and a $\bar{v}$ of 0.73 ml g^{-1}. Budzko et al. (1971) reported a value of 185 000 daltons for human C3, as estimated by a polyacrylamide gel electrophoretic method which is subject to an accuracy of only 5-10%.

Molar Extinction Coefficient of C3. An $\epsilon_{1cm}^{1\%}$ at 280 nm of 9.7 was determined for post-hydroxylapatite C3 in the ultracentrifuge observing boundary spreading with Raleigh interference optics and assuming a refractive index increment (Chervenka, 1969). Based upon an apparent weight average molecular weight of 187 650, a molar extinction coefficient of 1.82×10^5 l. mol^{-1} cm^{-1} at 280 nm was calculated. An ϵ_{276nm} of 1.66×10^5 l. mol^{-1} cm^{-1} was calculated from the amino acid composition shown in Table III using the extinction coefficients of 5400, 1500, 145, and 15 l. mol^{-1} cm^{-1} for tryptophan, tyrosine, cystine, and phenyalalanine, respectively (Donovan, 1969). These coefficients for C3 agree within 9-10%.

Amino Acid Analysis of Human C3. The amino acid composition of post-hydroxylapatite C3 is shown in Table III, calculated for a carbohydrate-free molecular weight of 185 000. A 1.5% (w/w) carbohydrate content (0.3% amino sugar and 1.2% neutral sugar, unpublished observations) has been determined for C3. The compositions for human C3 reported by Budzko et al. (1971) and Molenaar et al. (1974) are also given in Table III with appropriate corrections for comparative purposes. In general, the discrepancies are rather minor, although there is wide fluctuation in the values reported for half-cystine and methionine, which have inherent problems with quantitation. Comparisons of amino acid compositions of C3, as reported here and by others, must be viewed in the context of the shortcoming that no efforts have been made in any case to restrict the known genetic polymorphism (Alper and Propp, 1968). The compositions reported have no unusual or distinctive characteristics. Efforts in this laboratory to identify uncommon amino acids, such as hydroxyproline or hydroxylysine, were without success.

Carboxyl-Terminal Studies of C3. An effort to establish the carboxyl-terminal amino acids of post-hydroxylapatite C3 was undertaken as a structural parameter in defining the activation and degradation of the component. Previous attempts to identify the carboxyl-terminal amino acids of C3 by digestion with carboxypeptidases A and B at various pH's after heat denaturation, after reduction and alkylation, after complete succinylation, and in the presence of 6 M urea met with no success (Budzko et al., 1971). Similarly, our attempts to obtain release with CpA after reduction and alkylation were without success. However, if the digestions of reduced and alkylated C3 with CpA were carried out in the presence of 0.1% sodium dodecyl sulfate, a rapid release of alanine equivalent to 1 mol/mol of protein was seen. After prolonged digestion of C3 in 0.1% sodium dodecyl sulfate with a mixture of CpA/CpB, the yield of alanine reached 1.32 mol/mol of substrate. At no time was the release of any other residue observed.

When viewed in the context of the molecular structure of C3 as being composed of two constituent chains, the interpretation of this data is not unambiguous. Clearly, at least one polypeptide chain terminates with an alanine residue. Where greater than molar yields of alanine were observed it could arise from the penultimate residue of a terminal Ala-Ala sequence from one chain or represent the slow release of an alanine from the carboxyl terminus of the second chain. Attempts to decide between these alternative possibilities by the quantitative hydrazinolysis of C3 were unsuccessful.

N-Terminal Studies on Human C3. Where appropriate, the techniques of thin-layer chromatography, high-pressure liquid chromatography, and/or back-hydrolysis of DNP derivatives were employed to investigate the N terminus of human C3. In agreement with the observations of Budzko et al. (1971) and Molenaar et al. (1973), only DNP-serine could be identified

TABLE III: Amino Acid Composition of Human C3.[a]

	This Paper[b]	Budzko et al. (1971)[c]	Molenaar et al. (1974)[d]
Lysine	111.6	113.5	129.5
Histidine	27.7	28.3	17.5
Arginine	81.2	71.7	70.2
S-Aminoethyl cysteine	21.3	ND[e]	ND[e]
S-Carboxymethyl cysteine	21.6	ND[e]	ND[e]
Aspartic acid	144.9	162.5	160.5
Threonine	100.3	104.4	102.7
Serine	107.5	106.6	106.0
Glutamic acid	217.2	231.1	209.2
Proline	77.0	82.9	111.3
Glycine	97.0	105.0	97.1
Alanine	94.2	98.8	100.8
Half-cystine	ND[e]	38.6	8.4
Valine	141.8	146.9	139.7
Methionine	44.3	29.3	34.5
Isoleucine	77.6	72.1	75.6
Leucine	155.4	149.8	148.8
Tyrosine	56.2	58.1	58.3
Phenylalanine	60.1	61.2	60.6
Tryptophan	14.4	ND[e]	ND[e]
Total residues	1630	1661	1631

[a] Reported as mol of amino acid/mol of protein. [b] Calculated on the basis of a molecular weight for C3 of 187 650, corrected to 184 845 using 1.5% as the estimated carbohydrate. [c] Recalculated to molecular weight of 185 000 as original data erroneously used amino acid molecular weight rather than anhydrous residue wt. [d] Calculated on basis of molecular weight of 190 000, corrected to 184 490 using 2.9% as the estimated carbohydrate content. [e] Not determined.

TABLE IV: Edman Degradation of Human C3.[a]

Step	Residue Identified	Method of Identification[d]
1	Ser (5.1)[b]	HI, HPLC
2	Val (16.0)	GC, HI, HPLC
	Pro	GC, HI, HPLC
3	Glx (16.3)	HI, HPLC
	Met	GC, HPLC
4	Leu (24.3)	GC, HI, HPLC
	Tyr (14.6)	GC, HI, HPLC
5	Thr (28.7)[c]	HI
	Ser (12.3)[b]	HI
6	Glx (18.6)	HI
	Ile (16.7)	GC, HI
7	Lys (53.5)	HI
	Gly (12.6)	GC, HI
8	Arg	HI, TLC
	Thr (32.0)[c]	HI
9	Met	GC
	Pro (53.0)	GC, HI
10	Asx (28.8)	HI

[a] Percent recovery of residue is given in parentheses when quantitation was possible, based on Pth-norleucine internal standard. [b] Identified as alanine on back hydrolysis. [c] Identified as α-aminobutyric acid on back hydrolysis. [d] GC, gas chromatography; HI, back hydrolysis; TLC, thin-layer chromatography; HPLC, high-pressure liquid chromatography.

as the N-terminal residue of post-hydroxylapatite C3. Neither DNP-arginine nor DNP-histidine was seen upon examination of the aqueous phase after hydrolysis of dinitrophenylated C3. No DNP-cysteine derivatives or radioactivity could be identified where the C3 had been reduced and alkylated with [^{14}C]iodoacetic acid before dinitrophenylation. Finally, attempts to identify the presence of di-DNP-tyrosine or DNP-tryptophan by hydrolysis of dinitrophenylated C3 in 3 N mercaptoethanesulfonic acid were unfruitful. The recovery of DNP-serine from dinitrophenylated C3 ranged from 1.0 to 1.1 on a molar basis, uncorrected for hydrolytic destruction.

Reduced and alkylated C3 was examined in the JEOL sequenator, where it was possible to follow the development of two independent sequences. With the exception of the first position, two residues were identified in each position, as shown in Table IV. Only Pth-serine was seen in the first step, although back hydrolysis of the sample in HI released alanine in poor yield. Efforts to identify other Pth derivatives in this position were without success. Nor was radioactivity observed in the first 10–15 residues if the C3 was reduced and radioalkylated before being placed in the sequenator cup. When taken in conjunction with the high recovery of DNP-serine obtained in our earlier experiments, the tentative conclusion was reached that both polypeptide chains probably initiate with a serine residue. Methionines in positions 3 and 9 were clearly and repetitively established. Cleavage of the chains at these residues by the use of cyanogen bromide is currently being investigated as a means of isolating the N-terminal peptides.

Based upon these data, the N-terminal sequence of C3 is Ser-Val,Pro-Glx,Met-Tyr,Leu-Thr,Ser-Glx,Ile-Lys,Gly-Arg,Thr-Met,Pro-Asx. A sequence consistent with the N-terminal sequence of anaphylatoxin as reported by Hugli et al. (1975) can be derived from these data, and tentative sequences for each constituent chain of C3 thus postulated. However, the sequences were unequivocally established on the isolated chains themselves (Tack, B. F., Morris, S., and Prahl, J. W., in preparation). Clearly, both chains are amenable to N-terminal Edman degradation, and the β chain is not blocked as previously proposed (Budzko et al., 1971).

Acknowledgments

We express our appreciation to Carol Bolotin, Shelesa Brew, and Sam Morris for their technical assistance throughout the course of this work, and to Irene Thorell for her help with the preparation of this manuscript.

References

Alper, C. A., and Propp, R. P. (1968), *J. Clin. Invest. 47,* 2181–2191.

Bianco, C., Griffin, F. M., Silverstein, S. C., et al. (1975), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 34,* 956.

Bokisch, V. A., Dierich, M. P., and Müller-Eberhard, H. J. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 1989–1993.

Bokisch, V. A., Müller-Eberhard, H. J., and Cochran, C. G. (1969), *J. Exp. Med. 129,* 1109–1130.

Budzko, D. B., Bokisch, V. A., and Müller-Eberhard, H. J. (1971), *Biochemistry 10,* 1166–1172.

Budzko, D. B., and Müller-Eberhard, H. J. (1969), *Science 165,* 506–507.

Chervenka, C. H. (1969), A Manual of Methods for the Analytical Ultracentrifuge, Palo Alto, Calif., Spinco Division of Beckman Instruments.

Cohn, E. J., and Edsall, J. T. (1943), in Proteins, Amino Acids and Peptides, New York, N.Y., Reinhold, p 375.

Cooper, N. R., and Müller-Eberhard, H. J. (1970), *J. Exp. Med. 132,* 775–793.

Dalmasso, A. P. and Müller-Eberhard, H. J. (1964), *Proc. Soc. Exp. Biol. Med. 117,* 643–650.

DaSilva, W. D., Eisele, J. W., and Lepow, I. H. (1967), *J. Exp. Med. 116,* 1027–1048.

Deutsch, D. G., and Mertz, E. T. (1970), *Science 170,* 1095–1096.

Donovan, J. H. (1969), *Phys. Princ. Tech. Protein Chem., Part A,* pp 101–170.

Götze, O., and Müller-Eberhard, H. J. (1971), *J. Exp. Med. 134,* 905–1085.

Hartree, E. F. (1972), *Anal. Biochem. 48,* 422–427.

Hjertén, S. (1959), *Biochim. Biophys. Acta 31,* 216–235.

Hodgman, C. D. (1961), in Handbook of Chemistry and Physics, 45th ed, Cleveland, Ohio, The Chemical Rubber Publishing Co.

Huber, H., Polley, M. J., Linscott, W. D., Fudenberg, H. H., and Müller-Eberhard, H. J. (1968), *Science 162,* 1281–1283.

Hugli, T. E., Vallota, E. H., and Müller-Eberhard, H. J. (1975), *J. Biol. Chem. 250,* 1472–1478.

Itano, H. A., and Robinson, E. A. (1972), *J. Biol. Chem. 247,* 4819–4824.

Knott, G. D., and Shrafer, R. I. (1972), *Computer Graphics: Proceedings of the Siggraph Computers in Medicine Symposium 6,* 138–151.

Lay, W. H., and Nussenzweig, V. (1968), *J. Exp. Med. 128,* 991–1007.

Lee, J. C. and Timasheff, S. N. (1974), *Biochemistry 13,* 257–265.

Mancini, G., Carbonara, A. O., and Heremans, J. F. (1965), *Immunochemistry 2,* 235–254.

Mayer, M. M. (1961), in Experimental Immunology, Kabat, E. A., and Mayer, M. M., Ed., Springfield, Ill., Chas. C

Thomas, pp 149–153.
Miller, G. W., and Nussenzweig, V. (1974), *J. Immunol. 113,* 464–469.
Molenaar, J. L., Helder, A. W., Müller, M. A. C., Goris-Mulder, M., Jonker, L. S., Brouwer, M., and Pondman, K. W. (1974), in The Third Human Complement Component, Molenaar, J. L., Ed., Amsterdam, Graduate Press, pp 72–94.
Molenaar, J. L., Müller, M., and Pondman, K. W. (1973), *J. Immunol. 110,* 1570–1574.
Müller-Eberhard, H. J. (1975), *Annu. Rev. Biochem. 42,* 697–724.
Müller-Eberhard, H. J., Dalmasso, A. P., and Calcott, M. A. (1966), *J. Exp. Med. 123,* 33–54.
Müller-Eberhard, H. J., Nilsson, U., and Aronsson, T. (1960), *J. Exp. Med. 111,* 201–215.
Müller-Eberhard, H. J., Polley, M. J., and Calcott, M. A. (1967), *J. Exp. Med. 125,* 359–380.
Nilsson, U., and Mapes, J. (1973), *J. Immunol. 111,* 293–294.
Nilsson, U. R., and Müller-Eberhard, H. J. (1965), *J. Exp. Med. 122,* 277–298.
Nilsson, U. R., Tomar, R. H., and Taylor, F. B., Jr. (1972), *Immunochemistry 9,* 709–723.
Nishikawa, A. H., and Bailon, P. (1975), *Arch. Biochem. Biophys. 168,* 576–584.
Penke, B., Ferenczi, R., and Kovács, K. (1974), *Anal. Biochem. 60,* 45–50.
Pisano, J. J., and Bronzert, T. J. (1969), *J. Biol. Chem. 244,* 5597–5607.
Polson, A., Potgieter, G. M., Lergier, J. F., Mears, G. E. F., and Joubert, F. J. (1964), *Biochim. Biophys. Acta 82,* 463–475.
Prahl, J. W. (1976), in Trace Components of Plasma, Greenwalt, T. J., and Jamieson, G. A., Ed., New York, N.Y., Alan Liss, pp 43–64.
Sandberg, A. L., Osler, A. G., Shin, H. S., and Oliveira, B. (1970), *J. Immunol. 104,* 329–334.
Scheidegger, J. J. (1955), *Int. Arch. Allergy Appl. Immunol. 7,* 103–111.
Smithies, O., Gibson, D., Fanning, E. M., Goodfleisch, R. M., Gilman, J. G., and Ballantyne, D. L. (1971), *Biochemistry 10,* 4912–4921.
Summers, M. R., Smythers, G. W., and Oroszlan, S. (1973), *Anal. Biochem. 53,* 624–628.
Teller, D. C. (1973), *Methods Enzymol. 27,* 346–441.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406–4412.
Zimmerman, C. L., Pisano, J. J., and Appella, E. (1973), *Biochem. Biophys. Res. Commun. 55,* 1220–1224.

Fluorescence Depolarization Studies of Phase Transitions and Fluidity in Phospholipid Bilayers. 1. Single Component Phosphatidylcholine Liposomes†

B. R. Lentz,‡ Y. Barenholz, and T. E. Thompson*

ABSTRACT: The fluorescence depolarization associated with the hydrophobic fluorescent probe 1,6-diphenyl-1,3,5-hexatriene is used to monitor the changes in fluidity accompanying the gel–liquid crystalline phase transition in synthetic phosphatidylcholine dispersions. The parameters of the phase transition are determined for both large, multilamellar liposomes and small, single-lamellar vesicles. These parameters are compared with those obtained using other techniques. In addition, the data are interpreted in terms of two limiting molecular models, which in turn offer insight into the structural differences between multilamellar liposomes and small vesicles.

The fluidity of the lipid bilayer component of biological membranes has been shown by many studies to influence a variety of membrane functions. This parameter, which has been defined operationally by a number of different techniques, is dependent on both lipid composition and temperature. The most dramatic alteration in bilayer fluidity occurs as a result of the phase transition from the gel to liquid crystalline states exhibited by many lipid bilayer systems. Recent studies, summarized by Lee (1975), have suggested that some of the physiologically important properties of biological membranes may derive in part from lateral phase separation and from compressibility changes in the bilayer accompanying the phase transition.

In this paper and the following one, we report studies of the fluidity and of the phase transition characteristics in small, single-lamellar vesicles and multilamellar liposomes prepared from pure synthetic phospholipids and from mixtures of these compounds. A fluorescent probe molecule, 1,6-diphenyl-

† From the Department of Biochemistry, University of Virginia School of Medicine, Charlottesville, Virginia 22901. *Received March 23, 1976.* This investigation was supported by National Institutes of Health Research Grants GM-14628, and HL-17576. Barry Lentz was the recipient of a National Institutes of Health Post Doctoral Fellowship.

‡ Current address: Department of Biochemistry and Nutrition, University of North Carolina School of Medicine, Chapel Hill, North Carolina, 27514.

[1] Abbreviations used: DPH, 1,6-diphenyl-1,3,5-hexatriene; POPC, 1-palmitoyl-2-oleyl-3-*sn*-phosphatidylcholine; EDTA, ethylenediaminetetraacetic acid; DMPC, DPPC, DSPC, and DOPC are 1,2-dimyristoyl-, 1,2-dipalmitoyl-, 1,2-distearoyl-, and 1,2-dioleyl-3-*sn*-phosphatidylcholine, respectively; NMR, nuclear magnetic resonance; Tempo, 2,2,6,6-tetramethylpiperidinyl-1-oxy.