Purification and Structural Analysis of the Fourth Component of Human Complement†

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ABSTRACT: The fourth component of human complement (C4) has been purified in 20% yield from fresh plasma using as starting material the 5–12% poly(ethylene glycol) precipitate which had been depleted of plasminogen by an affinity adsorbent. Sequential ion-exchange chromatography on diethylaminoethylcellulose, QAE-Sephadex, and DEAE-Bio-Gel A resulted in C4 homogeneous by immunological criteria and by polyacrylamide gel electrophoresis, the last chromatographic step achieving separation of native from inactivated C4. Reduction with 20 mM dithiothreitol for 2 h at 37 °C in 0.25 M 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride, pH 8.6, effected cleavage of the interchain disulfide bonds. A three-chain structure for C4 was confirmed, and molecular weight estimates of 93 000 ± 9300, 75 000 ± 7500, and 30 000 ± 3000 determined for the α, β, and γ chains, respectively. The effects of known inactivators of C4 upon the chains of C4 were investigated, confirming that the inactivations by $C1\bar{s}$ and trypsin were accompanied by the fragmentation of the α chain. Inactivation of C4 by hydrazine, on the other hand, produced no detectable change in chain size. Separation of the chains was accomplished by gel filtration in the presence of 1 M acetic acid. Amino acid compositions of native C4 and the constitutive chains have been performed, and N-terminal sequences of the latter established by automated Edman degradation.

The effector functions following interaction of antibody with antigen are mediated by a complex of proteins, known as complement, acting in a sequential or "cascade" fashion. Activation of the fourth component of complement, i.e., C4, is accomplished by the proteolytic action of the $C1\bar{s}$ subunit of the C1 complex on the component (Lepow et al., 1956). The isolation of the C4 protein from human serum (Müller-Eberhard and Biro, 1963) has permitted the investigation of the C4 reaction mechanisms as well as of the physicochemical changes accompanying its activation. Following the action of $C1\bar{s}$ on C4, the cytolytic activity of the protein is mediated by its interaction with the cell membrane and the subsequent binding of C2 and activation of the remaining complement components, C3-9 (Müller-Eberhard and Lepow, 1965; Müller-Eberhard et al., 1967). Cooper and Müller-Eberhard (1968) have shown that activation of C4 results in the membrane binding of only 5–10% of the activated C4, the remaining 90–95% of the protein being found in solution in a form no longer capable of sustaining its role in the cytolytic activity of the complement complex.

The C4 protein, estimated to have a molecular weight of 2.1×10^5, is cleaved by $C1\bar{s}$ into two fragments, i.e., C4a and C4b, with approximate molecular weights of 1.5×10^4 and $1.9–2.0 \times 10^5$, respectively. While no biological function has been demonstrated thus far for the C4a fragment, the binding of activated C4 to the cell membrane is postulated to involve the C4b fragment. The ability of C4 to function in the "cascade" can be abolished upon treatment with ammonia (Gordon et al., 1926), hydrazine (Ecker et al., 1943), or hydroxylamine (Seifter et al., 1963). Changes in immunoelectrophoretic behavior in C4 have been described following treatment with hydrazine (Müller-Eberhard and Biro, 1963) or $C1\bar{s}$ (Müller-Eberhard, 1969). The ability of hydrazine or hydroxylamine to destroy lytic function is not restricted to C4, but also pertains to the complement components C3 and C5 (Müller-Eberhard, 1969; Budzko and Müller-Eberhard, 1969). Recently, Schreiber and Müller-Eberhard (1974) have reported that human C4 is composed of three constitutive chains of 9.3, 7.8, and 3.3×10^4 g/mol by the criteria of polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

The present report describes an improved preparative procedure for the purification of human C4 from plasma permitting the isolation of several hundreds of milligrams of protein. The chain structure observed by Schreiber and Müller-Eberhard (1974) has been confirmed and the constitutive chains were fractioned by gel filtration. Chains so prepared have been subjected to amino acid analysis and automated Edman degradation. Finally, the effects of hydrazine, $C1\bar{s}$, and trypsin on the chain structure of C4 have been investigated.

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. PEG[1]-4000 was purchased from Union Carbide, and microgranular DEAE-cellulose (Whatman DE-52) was a product of Reeve Angel. Sephadex G-200 and Sepharose-CL 4B were purchased from Pharmacia Chemicals. QAE-Sephadex (Q-50), EDTA, $PhCH_2SO_2F$, DFP, ϵ-amino-*n*-caproic acid, L-lysine, and proteins used as molecular-weight markers were purchased from Sigma. Human IgG was purchased from Cutter Labo-

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[1] Abbreviations used are: PEG, poly(ethylene glycol); EDTA, ethylenediaminetetraacetic acid; DFP, diisopropyl fluorophosphate; DTT, dithiothreitol; Pth, phenylthiohydantoin; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; DEAE, diethylaminoethyl.

ratories. DEAE-Bio-Gel A, polyacrylamide resin (P-300), and polyacrylamide gel electrophoresis reagents were purchased from Bio-Rad. Benzamidine hydrochloride was a product of Aldrich Chemical Co. Hemolysin and sheep blood were purchased from Flow Laboratories.

Hemolytic Assays and Titrations. The hemolytic activity of human C4 was determined by a modification of the one-step method reported by Gaither et al. (1974) using serum from a strain of C4-deficient guinea pigs. The colony was established through the auspices of the Small Animal Section of the Veterinary Resources Branch of the N.I.H. The C4 content of column eluants was qualitatively evaluated by an assay system containing a dilution of sample (equivalent to 0.001–0.01 μL), 0.1 mL of a 1:5 dilution of C4-deficient serum, 0.5 mL of 4.94 mM sodium Veronal buffer containing 142 mM NaCl, 0.1% gelatin, 0.3 mM $CaCl_2$, and 1.0 mM $MgCl_2$, and 0.4 mL of "sensitized" sheep erythrocytes (Mayer, 1961; at 1×10^8 cells/mL). After incubation at 37 °C for 10 min, the reaction was stopped by addition of 2 mL of cold isotonic saline, the unlysed cells were pelleted by centrifugation, and the absorbance at 415 nm was measured. C4 hemolytic activity was quantitated with 2×10^7 "sensitized" sheep erythrocytes in 2.47 mM sodium Veronal buffer containing 71 mM NaCl, 0.05% gelatin, 0.3 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 2.5% glucose following incubation at 37 °C for 1 h. The hemolytic unit, CH_{50}, is the reciprocal of the dilution of C4 that will lyse 1×10^7 "sensitized" sheep erythrocytes.

Antiserum and Immunological Techniques. Rabbit antisera to human C3, C4, IgG, IgA, IgM, fibrinogen, transferin, ceruloplasmin, α_2-macroglobulin, β-lipoprotein, and whole human serum were purchased from Behring Diagnostics. Goat anti-C5 was obtained from Meloy Laboratories, and goat anti-$C1\bar{s}$ from Kallestad. A guinea pig anti-human C4 was made in C4-deficient guinea pigs using post DEAE-Bio-Gel C4 as antigen. Equal volumes of protein (2 mg/mL) and complete Freund's adjuvant were emulsified, and a total of 1 mL per animal was inoculated subcutaneously. The animals were bled 1 month later.

Total C4 protein was quantitated by radial immunodiffusion according to Mancini et al. (1965). The plates were purchased from Behring Diagnostics. Immunoelectrophoresis in 1% agarose was performed according to Scheidegger (1955). Immunodiffusions were done on plates purchased from Hyland Laboratories.

Polyacrylamide Gel Electrophoresis. C4 separations were examined by gel electrophoresis according to the procedure of Weber and Osborn (1969). The monomer acrylamide concentration of the gels was 5.8% with an acrylamide/bisacrylamide ratio of 87:1. Samples were prepared by bringing them to a concentration of 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol and incubating at 80 °C for 2 min. *Escherichia coli* β-galactosidase, rabbit muscle phosphorylase, *Aspergillus niger* glucose oxidase, beef liver catalase, human IgG heavy chain, pig heart fumarase, yeast glyceraldehyde-3-phosphate dehydrogenase, human IgG light chain, and myoglobin from equine skeletal muscle were used as molecular weight markers, assuming values of 130 000, 100 000, 75 000, 58 000, 50 000, 49 000, 36 000, 23 500, and 17 200, respectively.

Inactivation of C4. C4 (3 mg/mL) was inactivated with $C1\bar{s}$ (isolated by the procedure of Valet and Cooper, 1974) by incubation of 0.6 mg of C4 with 0.047 mg of $C1\bar{s}$ at 37 °C for 1 h. Hydrazine inactivation was accomplished by incubation of C4 (3 mg/mL) with 0.3 M hydrazine at 37 °C for 1 h, followed by dialysis against phosphate-buffered saline at 4 °C. C4 (3 mg/mL) was inactivated with trypsin by incubation with 1% (w/w) β-trypsin for 1 min at room temperature; the reaction was stopped by addition of soybean trypsin inhibitor to a final concentration of 3% (w/w relative to trypsin). All samples were examined for hemolytic activity, immunoelectrophoretic mobility, and alterations in subunit structure (by gel electrophoresis in sodium dodecyl sulfate).

Isolation of the Chains of C4. Post-DEAE-Bio-Gel C4 (5.9 mg/mL) was dialyzed into 150 mM Tris-HCl buffer (pH 8.6) containing 5 mM EDTA, and was reduced by incubation with 20 mM DTT for 2 h at 37 °C. Alkylation was accomplished by addition of [^{14}C]iodoacetamide to 42 mM at 4 °C. The sample was first dialyzed into phosphate-buffered saline, and then into 1 M acetic acid. The chains were initially separated by gel filtration on a 2.5 × 160 cm column of Sephadex G-200 equilibrated in 1 M acetic acid. They were further purified by a second passage on the same column or by gel filtration on Sepharose-C4 4B equilibrated in 0.1 M ammonium bicarbonate containing 0.2% sodium dodecyl sulfate.

Amino Acid Analysis. Amino acid analyses were performed on a JEOL 60AH Amino Acid Analyzer coupled to an Autolab System AA Computing Integrator. Samples were hydrolyzed for 24, 48, and 72 h with two × 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 C4 chains was carried out on a JEOL Model 47K Sequence Analyzer using a modified double-cleavage program with 0.25 M Quadrol. Each chain pool (100–150 nmol) was fully reduced and alkylated with [^{14}C]iodoacetamide prior to sequence analysis. Known amounts of Pth-norleucine were placed in the fraction collector tubes or in the heptafluorobutyric acid as an internal standard. The thiazolinones were converted to the Pth-amino acid and identified on a Packard gas chromatograph on CFC (Pisano and Bronzert, 1969) and on the amino acid analyzer after back-hydrolysis with 5.7 N HCl containing 0.1% $SnCl_2$ (Mendez and Lai, 1975). For identification of particular residues, thin-layer chromatography (Summers et al., 1973), and the arginine stain of Yamada and Itano (1966) were employed. The use of [^{14}C]iodoacetamide to alkylate cysteine residues permitted the identification of the *S*-carboxymethylcysteine by virtue of the radioactivity.

Purification Procedure. All operations were performed at 4 °C.

Step 1: Fractionation of Plasma with PEG. Twelve units of freshly drawn plasma were chilled individually in an ice bath and made 0.5 mM in $PhCH_2SO_2F$ by addition of an anhydrous 2-propanol solution of $PhCH_2SO_2F$ (20 mg/mL). Each unit was made 5% in PEG by slow addition, with stirring, of 15% (w/v) PEG in a 100 mM sodium phosphate buffer (pH 7.4), containing 150 mM NaCl, 15 mM EDTA, and 0.5 mM $PhCH_2SO_2F$. After a 30-min incubation period, the precipitate was removed by centrifugation at 5000*g* for 20 min and the supernatants were pooled. The PEG concentration was increased to 12% by slow addition, with stirring, of 26% (w/v) PEG in the same buffer as above. After 30 min, the precipitate was collected by centrifugation at 7000*g* 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. It was then made 10 mM in DFP by addition of an anhydrous 2-propanol solu-

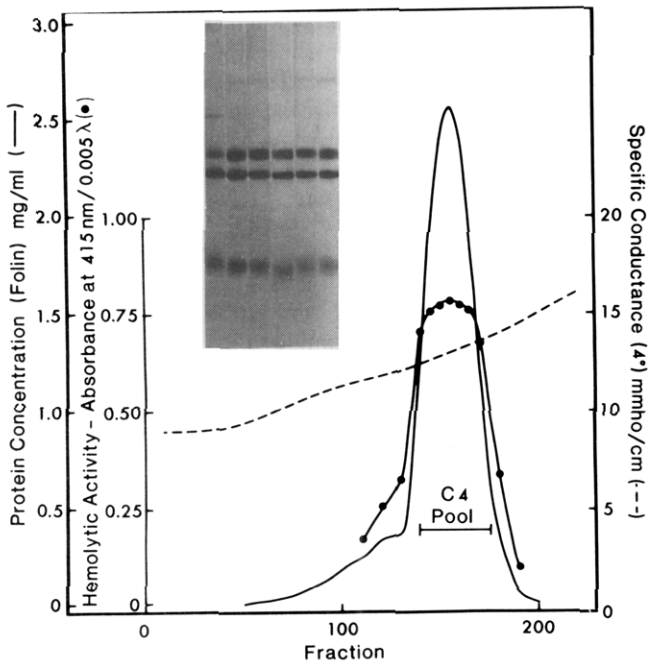

FIGURE 1: Ion-exchange chromatography on a column of QAE-Sephadex (2.4 × 40 cm) of the post-DEAE-cellulose pool of human C4 obtained from the plasminogen-depleted 5–12% PEG fraction of 12 units of fresh human plasma. The column had been equilibrated with 25 mM sodium phosphate buffer (pH 7.4) containing 100 mM NaCl, 100 mM ϵ-amino-*n*-caproic acid, 25 mM benzamidine hydrochloride, and 5 mM EDTA. The sample was adjusted to pH 7.4 and specific conductance of 9.2 mmho/cm (4 °C) and loaded on the column. After a wash with the starting buffer, a linear gradient of NaCl from 100 to 300 mM (1.4 L total) was developed. Absorbance at 280 nm (—), specific conductivity (- - -), and hemolytic activities of C4 (●–●) are shown. C4-containing fractions were pooled as indicated. The insert from left to right shows the patterns of fraction's 135, 145, 155, 165, and 175 upon gel electrophoresis in the presence of sodium dodecyl sulfate and mercaptoethanol.

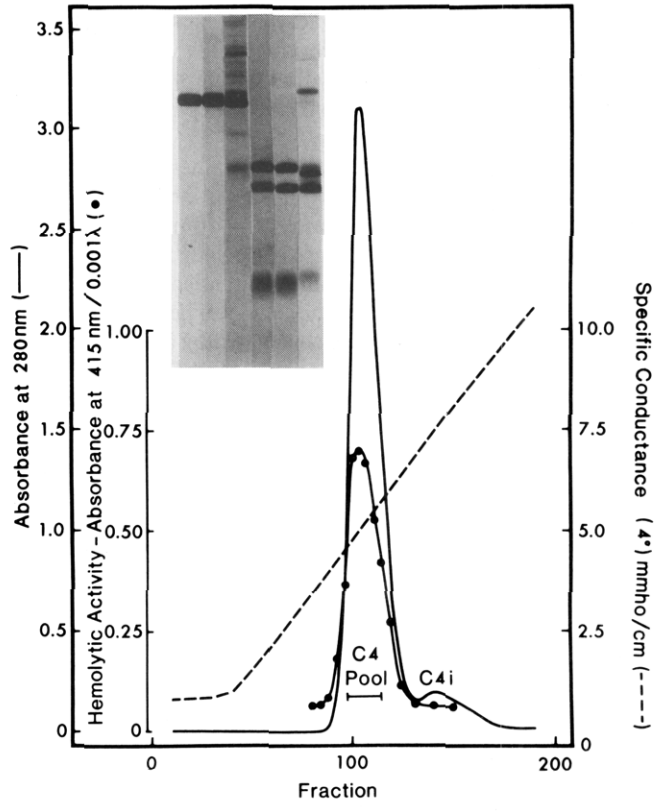

FIGURE 2: Ion-exchange chromatography of the post-QAE C4 pool on a 2.5 × 25 cm column of DEAE-Bio-Gel A. The column had been equilibrated with 10 mM Tris-HCl buffer (pH 8.0) containing 50 mM ϵ-amino-*n*-caproic acid and 5 mM EDTA. The sample, after dialysis against the equilibration buffer, was applied to the column and a linear gradient of NaCl to 200 mM (1 L total) was developed. Absorbance at 280 nm (—), specific conductivity (- - -), and hemolytic activity of C4 (●–●) are shown. The C4-containing fractions were pooled as indicated. The insert, from left to right, shows the patterns of fractions 100, 112, and 142 upon gel electrophoresis in sodium dodecyl sulfate, first in the absence and then in the presence of mercaptoethanol.

tion of DFP (100 mg/mL).

Step 2. Plasminogen Depletion of the 5–12% PEG Fraction. A 5 × 20 cm column of L-lysine–polyacrylamide prepared according to Rickli and Cuendet (1971) was equilibrated with a 100 mM sodium phosphate buffer (pH 7.4) containing 150 mM NaCl and 5 mM EDTA. The 5–12% PEG fraction was passed through the column, followed by several hundred milliliters of the equilibration buffer.

Step 3. DEAE-Cellulose Chromatography. The plasminogen-depleted 5–12% PEG fraction was diluted to a specific conductance of 3.0 mmho/cm (4 °C) with a solution of 100 mM ϵ-amino-*n*-caproic acid and 5 mM EDTA, and the pH was adjusted to 7.0. The sample was applied to a 5 × 160 cm column of DEAE-cellulose equilibrated with 25 mM potassium phosphate buffer (pH 7.0) containing 100 mM ϵ-amino-*n*-caproic acid and 5 mM EDTA. The sample was followed with 3.2 L of starting buffer, and finally a linear NaCl gradient (total volume of 8 L) was developed to a limiting concentration of 300 mM NaCl. The flow rate was maintained at 125 mL/h, 22-mL fractions were collected, and the absorbance at 280 nm was monitored. C4 was found to elute at a specific conductance of 7.5–8.5 mmho/cm (4 °C), and a C4 pool was made on the basis of hemolytic activity (see Figure 1 of Tack and Prahl, 1976).

Step 4. QAE-Sephadex Chromatography. The post-DEAE C4 pool was made 0.5 mM in $PhCH_2SO_2F$, incubated 1 h, and then made 25 mM in benzamidine hydrochloride by the addition of solid. The conductivity was adjusted to 9.2 mmho/cm (4 °C), and the pH to 7.4. The sample was applied to a 2.5 × 40 cm column of QAE-Sephadex equilibrated in 25 mM sodium phosphate buffer (pH 7.4) containing 100 mM NaCl, 100 mM ϵ-amino-*n*-caproic acid, 25 mM benzamidine hydrochloride, and 5 mM EDTA. The sample was followed with the starting buffer, and the C4 finally eluted by development of a linear NaCl gradient (total volume of 1.4 L) to a limiting concentration of 300 mM NaCl. Six-milliliter fractions were collected at a flow rate of 35 mL/h. Protein concentration was determined by the Lowry method (Lowry et al., 1951), except that three times the normal concentration of $CuSO_4$ was used to compensate for the presence of EDTA in the buffer. C4 eluted at a specific conductance of 13 mmho/cm (4 °C). The fractions containing C4 were identified and pooled on the basis of hemolytic activity (Figure 1).

Step 5. DEAE-Bio-Gel A Chromatography. The post QAE-Sephadex C4 pool was dialyzed against a 10 mM Tris-HCl buffer (pH 8.0) containing 50 mM ϵ-amino-*n*-caproic acid and 5 mM EDTA and was applied to a 2.5 × 35 cm column of DEAE-Bio-Gel A equilibrated in the same buffer. The sample was followed with starting buffer at a flow rate of 30 mL/h, and a linear NaCl gradient (total volume of 1 L) was developed to a limiting concentration of 200 mM NaCl. Five-milliliter fractions were collected, and the absorbance at 280 nm was monitored. C4 eluted at a specific conductance of 5 mmho/cm (4 °C); the fractions containing C4 hemolytic activity were pooled on the basis of purity as determined by gel electropho-

TABLE I: Summary of C4 Purification.

Step	Total C4[a] (mg)	Yield (%)	Total C4[b] (CH_{50} Units)	Yield (%)	CH_{50} Units C4/ mg of Total Protein[c]	Fold Purification
Plasma	1043	100	9.56×10^9	100	4.72×10^4	1
5-12% PEG ppt	540	52	5.67×10^9	59	1.19×10^5	2.5
L-lysine adsorb.	573	55	5.31×10^9	56	1.14×10^5	2.4
DEAE-cellulose	530	51	3.77×10^9	39	4.31×10^6	91
QAE-Sephadex	339	33	2.58×10^9	27	7.32×10^6	155
DEAE-Bio-Gel A	227	22	1.91×10^9	20	8.41×10^6	178

[a] Determined by the Mancini method of radial immunodiffusion (Mancini et al., 1965). [b] Determined by hemolytic titration using EA cells and C4-deficient guinea pig serum as a source of other complement components. [c] Protein determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

resis in sodium dodecyl sulfate (Figure 2). The C4 pool was concentrated by applying it to a 2.5 × 5 cm column of QAE-Sephadex equilibrated with 25 mM sodium phosphate buffer (pH 7.4) containing 100 mM NaCl and 5 mM EDTA, and eluting it with the same buffer adjusted to contain 400 mM NaCl.

Results and Discussion

Comments on the Purification. The isolation of human C4 was developed in conjunction with the purification scheme of C3 reported by Tack and Prahl (1976) to make maximal use of natural resources. The use of PEG to fractionate human plasma has not led to inactivation of either C4 or C3. Whereas the 5-12% PEG fraction contains approximately 80% of the total C3, only about 60% of the C4 is found in this fraction (Table I). Raising the final PEG concentration above 12% did not significantly increase the yield of C4. Indeed, it was suspected that precipitates in excess of 12-14% PEG contained inactivating substances as we experienced poorer final recoveries of hemolytically active C4 using these than with the 5-12% PEG fraction. Finally, whereas both fresh human plasma and frozen fresh human plasma appeared to be suitable sources for the purification of human C3, such was not the case for C4. The use of frozen fresh plasma (−70 °C) was marked by erratic recoveries of hemolytically active C4.

As described in the purification of C3 (Tack and Prahl, 1976) depletion of plasminogen by the biospecific affinity adsorbent L-lysine-agarose was complicated by weak ion-exchange properties of the gel. These problems were markedly diminished by replacing the agarose matrix with polyacrylamide, and coupling the lysine via the azide derivative according to Rickli and Cuendet (1971). On DEAE-cellulose chromatography, the C4 eluted after the C3/C5 peak (Figure 1 of Tack and Prahl, 1976). Ceruloplasmin usually was seen on the ascending limb of the C4 peak, and, while it was a useful marker, it also posed as a potential contaminant. The post-DEAE C4 pool represented 51% of the initial plasma C4 protein, but only 39% of the hemolytic activity (Table I), indicative of activation of the C4 before or during the chromatographic step. The source of the inactivation is not clear, although we were able to identify $C1\bar{s}$ immunochemically upon concentration of the pool.

Indeed, the concentration of the pool, ranging from 1.0 to 1.5 L in volume, was a formidable obstacle. Efforts to precipitate the C4 by PEG or ammonium sulfate were unsuccessful, due to the low concentration of protein in the pool. Concentration by ultrafiltration was accompanied by partial or complete loss of C4 hemolytic activity. The C4 could be effectively adsorbed on QAE-Sephadex at conductivities at which contaminating proteins were unretarded (Figure 1). Thus, both concentration and further purification could be achieved simultaneously, as indicated by examination of the fractions using gel electrophoresis in sodium dodecyl sulfate. Benzamidine, a competitive inhibitor of trypsin-like proteases (Mares-Guia and Shaw, 1965), was introduced at this step in an effort to minimize the loss of C4 hemolytic activity. Gel filtration of the post-QAE C4 on Sepharose 6B revealed only a single peak and therefore was not routinely employed, although it may be considered where contaminants significantly larger or smaller than C4 are encountered.

Resolution of hemolytically inactive C4 from native C4 was accomplished by chromatography of the post-QAE C4 pool on DEAE-agarose (Figure 2). Whereas C4 protein could be identified in both the first and second peaks by immunodiffusion against anti-C4 antiserum, the C4 of only the first peak was capable of inducing lysis in the assay system. Gel electrophoresis in sodium dodecyl sulfate of a fraction in the second peak revealed degradation of the α-chain band (see insert, Figure 2), a change characteristically associated with the action of $C1\bar{s}$ on the C4 protein. The post DEAE-Bio-Gel A pool accounted for 22% of the initial plasma C4 antigenic activity and 20% of the initial C4 hemolytic activity (Table I). The hemolytic activity of this C4 was determined to be 8.4×10^6 CH_{50} units/mg of protein. This C4 was found to remain stable for at least 6 months when stored at −70 °C.

Criteria of Purity. Post DEAE-Bio-Gel C4 was found to be free of contaminants by immunodiffusion at 0.7 mg/mL and 7.0 mg/mL of C4 against anti-$C1\bar{s}$, C3, C5, IgG, IgM, IgA, ceruloplasmin, fibrinogen, transferin, β-lipoprotein, α_2-macroglobulin, and whole human serum. Only one precipitin line was observed upon immunodiffusion or immunoelectrophoresis of C4 using antipost DEAE-Bio-Gel C4 produced in a C4-deficient guinea pig. Radial immunodiffusion revealed a trace contamination of the C4 by IgG at less than 0.4% (w/w). When examined by gel electrophoresis in the presence of sodium dodecyl sulfate, the unreduced C4 gave a single band, while the reduced C4 gave a pattern of three bands (vide infra).

Polyacrylamide Gel Electrophoresis Analysis. In agreement with the report of Schreiber and Müller-Eberhard (1974), human C4 appears to contain three constitutive chains upon gel electrophoresis in the presence of sodium dodecyl sulfate and DTT. The molecular weights of the α, β, and γ chains were estimated by us to be 93 000 ± 9300, 75 000 ± 7500, and 30 000 ± 3000, respectively, accounting for a total C4 molecular weight of 198 000. Considering the inherent error of the method, this again is in general agreement with Schreiber and Müller-Eberhard (1974). Using the molecular

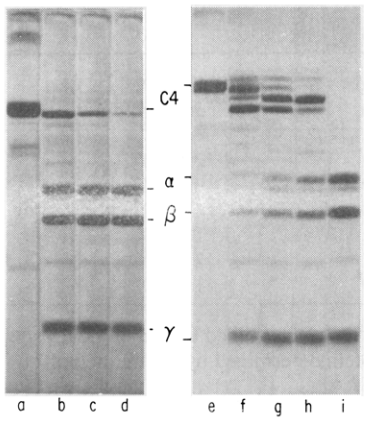

FIGURE 3: The reduction of human C4 at pH 8.6 and 7.2. The C4 (1 mg/mL) was dialyzed into 250 mM Tris-HCl (either pH 8.6 or 7.2) containing 5 mM EDTA and incubated for 2 h at 37 °C at various dithiothreitol concentrations. Reduction was stopped by the addition of iodoacetamide, and the samples were dialyzed into saline and then 0.1% sodium dodecyl sulfate containing iodoacetamide. Electrophoresis on polyacrylamide was performed according to Weber and Osborn (1969). Gels b through d were reduced at pH 8.6, and f through i at pH 7.2. From left to right: a and e, unreduced C4; b and f, C4 with 5 mM DTT; c and g, with 10 mM DTT; d and h, with 20 mM DTT; i with 20 mM DTT in the presence of 0.1% sodium dodecyl sulfate.

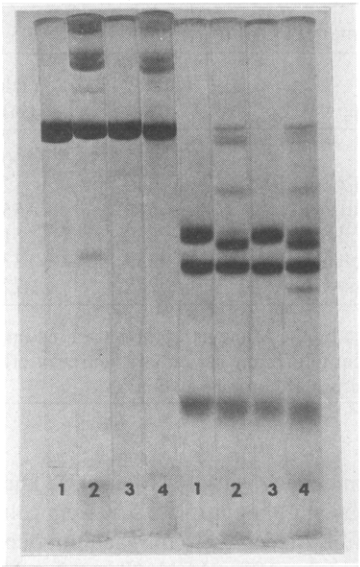

FIGURE 5: Studies of the chain structure in human C4 after inactivation by various reagents as revealed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis in the absence (left gels 1–4) and presence (right gels 1–4) of mercaptoethanol. The samples shown are: gel 1, native C4; gel 2, C1$\bar{s}$-inactivated C4; gel 3, hydrazine-inactivated C4; and gel 4, trypsin-inactivated C4.

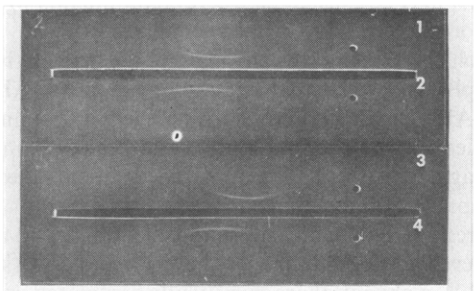

FIGURE 4: Immunoelectrophoretic analysis of native C4 (well 1), C1$\bar{s}$-inactivated C4 (well 2), hydrazine-inactivated C4 (well 3), and trypsin-inactivated C4 (well 4) developed against guinea pig anti-human C4. The cathode is shown to the right.

weights estimated and assuming the uptake of stain to be directly proportional to the mass of the chains, a ratio of α:β:γ of 0.69:0.86:1.00 was calculated from a densitometric trace of the stained gel.

The constitutive chains of C4 do not dissociate in the absence of reducing reagent (Figure 3, gel b), implying the quaternary structure of the native protein is stabilized by interchain disulfide bonds. Essentially all the interchain disulfide bonds are reduced at DTT concentrations of 20 mM (Figure 3, gels b–d) at pH 8.6. The reduction at this pH appears to be an "all or nothing" phenomenon, as no intermediate chain dimers are seen at this pH. If the reduction is performed at pH 7.2, however, the pattern becomes far more complex with the suggestion that the interchain bonds of the γ chain might be the first to be reduced (Figure 3, gels f–h). This is being further evaluated.

Inactivation of C4. The conversion of native C4 to hemolytically inactive C4 by treatment with C1$\bar{s}$, with trypsin, and with hydrazine was examined. C4 hemolytic activity was destroyed upon incubation with C1$\bar{s}$ or hydrazine, but only partially lost upon incubation with 1% trypsin for 1 min. In agreement with previous reports in the literature, C4 inactivated by C1$\bar{s}$ migrated more anodally than native C4 on immunoelectrophoresis (Müller-Eberhard and Lepow, 1965; this paper, Figure 4, well 2), while C4 inactivated by the action of hydrazine migrated more cathodally (Müller-Eberhard and Biro, 1963; this paper, Figure 4, well 3). C4 could also be inactivated by a tenfold lower concentration of hydrazine; i.e., 15 mM, but now demonstrated a slight anodal rather than cathodal shift. The immunoelectrophoretic mobility of C4 inactivated by the action of trypsin appeared to be the same as that of native C4 (Figure 4, well 4).

The chain structure of each type of inactivated C4 was examined by sodium dodecyl sulfate gel electrophoresis in the absence and in the presence of 20 mM DTT. C4 inactivated by C1$\bar{s}$ (Figure 5, gels 2) as well as that inactivated by trypsin (Figure 5, gels 4) revealed a characteristically smaller α chain, i.e., α' chain, with no obvious changes in the β or γ chains. This α' chain was estimated to possess a molecular weight of 8.6–8.7 $\times 10^4$, implying a decrease in molecular weight of only 6000–7000. This is considerably smaller than the molecular weight estimate of 15 000 reported by Budzko and Müller-Eberhard (1970) for the C4a fragment released by the action of C1$\bar{s}$ on C4 in their hands. In the studies reported here, no effort was made to identify or isolate any fragments. Both C1$\bar{s}$ and trypsin-treated C4 gave rise to bands migrating more slowly than the undissociated proteins or α-chains, which presumably represent aggregates as they were not seen in untreated (Figure 5, gels 1) or hydrazine-treated C4 (Figure 5, gels 3). Finally, bands due to further degradation of the α' chain were seen between the β and γ bands in the trypsin digests of C4.

It is not clear at this point why the electrophoretic mobilities of C1$\bar{s}$- and trypsin-inactivated C4 are not the same in view of the similar patterns they give on gel electrophoresis in sodium dodecyl sulfate. This may reflect subtle differences in cleavage site with the C4a peptide remaining bound to the residual C4 fragment by interchain disulfides in the case of trypsin inactivation. The absence of any detectable alteration of chain size in the hydrazine-inactivated C4 was unexpected (Figure 5, gels 3) in view of the pronounced change in electrophoretic behavior observed (Figure 4, well 3). This same disparity between the immunoelectrophoretic behavior and apparent absence of al-

TABLE II: Amino Acid Compositions of Human C4 and Constitutive Chains.[a]

Amino Acid	C4[b]	C4[c]	α	β	γ	$\frac{\alpha+\beta+\gamma}{C4}$
Lys	99.5	79.6	32.3	32.7	15.0	1.01
His	46.0	41.0	15.9	15.5	4.9	0.89
Arg	93.1	109.4	43.1	33.4	21.3	0.89
Asp	148.1	142.1	77.2	53.3	17.6	1.04
Thr	106.9	92.4	47.8	32.6	11.1	0.99
Ser	154.1	145.0	74.0	76.0	16.6	1.15
Glu	180.0	204.4	104.8	65.5	36.9	1.01
Pro	96.1	101.0	34.2	44.3	11.9	0.89
Gly	143.4	130.1	61.0	52.5	16.6	1.00
Ala	124.6	142.7	77.2	42.3	21.3	0.99
Cys	47.3	31.2[d]	20.4[d]	7.8[d]	7.7[d]	1.15
Val	136.9	137.2	55.7	66.8	22.0	0.97
Met	27.3	29.2	18.9	15.8	4.3	1.33
Ile	59.6	57.1	25.5	25.7	6.2	1.01
Leu	208.1	209.5	122.5	87.9	27.4	1.14
Tyr	49.0	54.3	15.9	20.4	12.1	0.89
Phe	77.7	65.7	21.5	27.6	11.2	0.92
Trp	ND[e]	19.6	10.7	ND[e]	0.9	
Total	1798	1792	859	689	265	1.01

[a] Mean of analyses of two preparations based upon assumed molecular weights of 198 000, 93 000, 75 000, and 30 000 for C4, α, β, and γ chains, respectively. [b] Data of Budzko and Müller-Eberhard (1970) recalculated to molecular weight of 198 000 for comparative purposes. [c] This paper. [d] Determined as *S*-carboxymethylcysteine. [e] Not determined.

teration of chain size on gel electrophoresis in sodium dodecyl sulfate has been observed with hydroxylamine-inactivated C3 (J. Prahl, unpublished observations). Thus, the mechanism for amine inactivation of C4 (and C3) remains obscure at this time, but may represent smaller proteolytic cleavages than detectable by gel electrophoresis in sodium dodecyl sulfate, alteration in carbohydrate content, or conformational changes, any or all resulting in the loss of hemolytic activity.

Isolation of the Chains of C4. The ability to reduce the interchain disulfide bonds in the absence of denaturing agents suggested the possibility of utilizing chain-separation methodology which had proved successful with the immunoglobulins. As shown in Figure 6, it was possible to fractionate the three chains on Sephadex G-200 in the presence of 1 M acetic acid, although the resolution was incomplete. Both C4 β and γ chains could be satisfactorily purified by appropriate pooling on the basis of gel electrophoresis in sodium dodecyl sulfate, concentration by ultrafiltration, and repeated filtration on the same gel.

The α chain of C4 proved to be a more intractable problem. The presence of β and γ chains in the α-chain peak (see Figure 6) could not represent residual unreduced C4, since free chains and not C4 were seen upon gel electrophoresis in sodium dodecyl sulfate of material from the α-chain peak in the absence of reducing reagent. Repetitive gel filtration of the α chain resulted in further, but not complete, purification. Propionic acid proved no more effective, and perhaps even less effective, than acetic acid in dissociating the chains. The final purification of the α chain was achieved by gel filtration on Sepharose-CL 6B in 0.1 M ammonium bicarbonate containing 0.2% sodium dodecyl sulfate. These conditions successfully disrupted the intrachain noncovalent interactions of chains. These observations suggest that the α-chain peak obtained from Sephadex G-200 in 1 M acetic acid may well represent aggregates formed by the self-association of α chain, the aggregates then binding or trapping β and γ chains. Unfortunately, filtration of α chain on Sepharose 6B in 1 M acetic acid led to complete adsorption of the material to the gel matrix, presumably by interaction with the residual charged sulfate groups on the matrix.

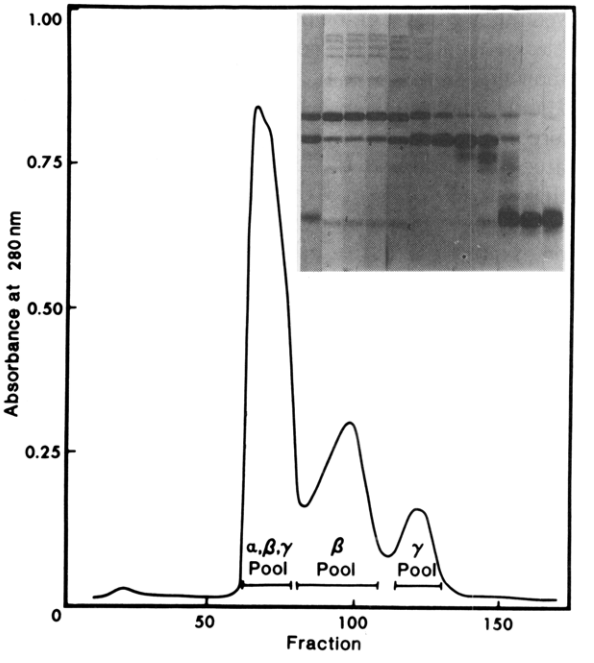

FIGURE 6: Gel filtration of reduced human C4 on a 2.5 × 160 cm column of Sephadex G-200 equilibrated and developed with 1 M acetic acid. The sample had been reduced in 250 mM Tris-HCl (pH 8.6) containing 20 mM dithiothreitol at 37 °C for 2 h. Reduction was stopped with iodoacetamide, and the sample was dialyzed into 1 M acetic acid. Absorbance at 280 nm is shown. The insert, from left to right, shows the gel electrophoretic patterns in sodium dodecyl sulfate of the starting material and fractions 66, 70, 74, 78, 82, 86, 98, 106, 112, 122, and 128.

Amino Acid Analysis of C4 and C4 Chains. The amino acid composition of post DEAE-Bio-Gel A C4 is shown in Table II calculated for a molecular weight of 198 000. This molecular

TABLE III: N-Terminal Sequences of the Chains of Human C4.[a]

	5	10	15	20
α	Asn-Val-Asn-Phe-Glu-Lys-Ala-Ile-Asn-Glu-Lys-Leu-Gly-Glu-Tyr-Ala-Ser-Pro-Thr-Ala-Lys			
β	Lys-Pro-Gly-Leu-Leu-Leu-$\frac{\text{Phe}}{\text{Leu}}$-$\frac{\text{X}}{\text{Phe}}$-Cys			
γ	Glx-Ala-Pro-Lys-Val-Val-$\frac{\text{His}}{\text{Glx}}$-Glx-Glx-Glx-Ser-X-Val-X-Tyr-Thr-Val-Cys-Cys-X-Gly			

[a] Derived from the automated Edman degradation of chains. Methods of identification and quantitative data are available as supplementary material.

weight of C4 was chosen as representing the summation of the molecular weights of the constitutive chains estimated by mobility on gel electrophoresis in sodium dodecyl sulfate. Although native C4 contains 1.5–2.0% carbohydrate, no effort was made to quantitate the carbohydrate content of the respective chains. The relative residue values expressed in Table II must be considered tentative, therefore, pending quantitative estimation of the carbohydrate contribution to the molecular weights. The composition of human C4 determined by Budzko and Müller-Eberhard (1970) has been recalculated to a molecular weight of 198 000 and reported in Table II for comparative purposes. The data are in general agreement, but show some divergence for residues such as lysine, arginine, glutamic acid, alanine, cysteine, and phenylalanine. The compositions reported have no unusual or distinctive characteristics, and the search for unusual amino acids, such as hydroxyproline or hydroxylysine, were without success. These data must be viewed in the context of the possible complicating existence of genetic polymorphism (Azen and Cooper, 1971).

Shown in Table II are the amino acid compositions of the α, β, and γ chains of C4 isolated and purified by gel filtration. Before analysis some samples were totally reduced and alkylated to determine the cysteine content as *S*-carboxymethylcysteine. The sum of the amino acid composition for the three chains is compared residue by residue with the composition of intact C4. In the case of some residues, i.e., histidine, arginine, proline and phenylalanine, a discrepancy of almost 10% was observed. Nonetheless, provocative differences between the compositions of the individual chains are obvious. The molar content of hydrophilic residues, i.e., threonine plus serine, varies from 21 to 16 to 10% for the α, β, and γ chains, respectively. Although the molar content of acidic residues, i.e., aspartic plus glutamic acids, is relatively constant at 21, 17, and 21%, the content of basic residues, i.e., lysine plus arginine, increases from 9 to 10 to 14% for the α, β, and γ chains, respectively. Clearly, the latter comparisons do not take into account the possible amidination of the acidic groups, which might exaggerate or negate what appear to be potentially significant charge differences between the chains.

N-Terminal Sequences of the Constituent Chains of Human C4. The isolated chains were totally reduced and radiolabeled with [^{14}C]iodoacetamide and subjected to automated Edman degradation commencing with 100–150-nmol samples. The derived sequences of at least duplicate determinations of each chain are shown in Table III. Nagasawa et al. (1976) have reported the N termini of both the α and γ chains of native C4 initiate with glutamic acid or glutamine, but were unable to identify an N-terminal residue for the β chain. While we confirm this observation upon sequencing the γ chain, the α chain in our hands clearly initiates with an asparagine residue. The sequences of both α and γ chains were discernible with only several ambiguities to at least 20 residues, with initial recoveries of 70 and 55%, respectively. The β chain of C4 was also accessible to sequencing, although recoveries of initial residues were below 40% and decreased rapidly in subsequent steps. A very low recovery of glycine was observed in step 3 of the β-chain sequence, which may well reflect the presence of a degraded tryptophan in this position. Beyond step 8 no Pth derivatives were clearly identifiable. At this time, we are not sure if these unidentifiable residues are the result of low yield or represent difficult identifications.

No homology is evident at this point among the N-terminal sequences of the chains of human C4. Nor is homology evident upon comparison either with the N-terminal sequences of the chains of human C3 (Tack and Prahl, 1976, and Tack et al., in preparation), or with the N terminus of the C3a anaphylactic fragment of human C3 (Hugli, 1975) or the C5a anaphylactic fragment of human C5 (Fernandez and Hugli, 1976). It is clear, however, that the constitutive chains of human C4 are amenable to N-terminal degradation, and this fact should assist in the definition of the molecular parameters of activation of the component. These studies and the delineation of the carboxyl-terminal structures of the chains are in progress.

Recent investigations have suggested that the structural and/or regulatory gene(s) controlling the expression of C4, in addition to C2, C8, and properdin factor B, are encoded in the histocompatibility gene complex in man (HLA; Teisberg et al., 1976) and in the mouse (H-2; Meo et al., 1975). As the products of this closely linked group of genes appear to be functionally involved in the phenomenon of immune recognition and response, the significance of the association of C4 with the complex is provocative, both in terms of its evolution as well as its possible involvement in these phenomena. A comparison of the N-terminal sequences of the chains of C4 with the partial sequences reported for the HLA products, i.e., the HLA-A and HLA-B antigens, again reveals no evident homology (Terhorst et al., 1976; Bridgen et al., 1976). When they become available, a comparison with the N-terminal sequences of the products of the immune response genes, i.e., the Ia antigens, could be of interest.

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Supplementary Material Available

A table indicating the method of identification and quantification of Pth derivatives (1 page). Ordering information is given on any current masthead page.

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