# Fifth Component of Human Complement: Purification from Plasma and Polypeptide Chain Structure†

Brian F. Tack,‡ Sam C. Morris, and James W. Prahl*

ABSTRACT: Human C5 has been isolated from fresh plasma utilizing an approach which also provides for the copurification of human C3 [Tack, B. F., & Prahl, J. W. (1976) *Biochemistry 15*, 4513–4521]. Initial fractionation of plasma with poly(ethylene glycol) followed by depletion of plasminogen on Sepharose–L-lysine, chromatography on diethylaminoethylcellulose, gel filtration on Sepharose 6B, chromatography on hydroxylapatite, and depletion of IgG subclasses 1, 2, and 4 on protein A–Sepharose proved satisfactory for obtaining C5 in high yield and biochemical purity. Final recoveries were 32% of the initial C5 protein and 29% of the initial C5 hemolytic activity as quantitated by specific immunoprecipitant and hemolytic titration assays. Apparent homogeneity was indicated by monodisperse behavior on immunoelectrophoresis and polyacrylamide gel electrophoresis. Total reduction and alkylation of disulfide bonds revealed a two-polypeptide chain structure: an $\alpha$ chain and a $\beta$ chain with respective molecular weights of 115 000 ± 12 000 and 75 000 ± 8000. A preparative separation of the chains was obtained by gel filtration on a Sepharose CL-4B column equilibrated with 0.2% sodium dodecyl sulfate in 0.1 M sodium bicarbonate (pH 7.9). Automated Edman degradation of C5 gave a single N-terminal sequence of Thr-Leu-Gln-Lys-Lys-Ile-Glx-Glx-Ile-Ala. An identical N-terminal sequence was observed with the isolated $\alpha$ chain. Several attempts to sequence the N-terminal region of the $\beta$ chain have been unsuccessful. Digestion of each chain with carboxypeptidases A and Y has indicated a common carboxyl-terminal sequence of (Ala,Val)-Ala-Gly-Ser. The identity in N-terminal structure between the C5 $\alpha$ chain and the C5a anaphylatoxin (Fernandez & Hugli, 1976) provides direct evidence that this potent anaphylactic and chemotactic polypeptide is released from the amino-terminal region of this chain.

The fifth component of human complement (C5) was first isolated and characterized as a discrete protein by Nilsson & Müller-Eberhard (1965). The method of purification was later refined (Nilsson et al., 1972) with particular emphasis placed on reducing the time required for its isolation and on the removal of plasminogen thereby significantly increasing the stability of the final product.

Activation of C5 by the "classical" pathway $\overline{C4b,2a,3b}$ enzyme (Cooper & Müller-Eberhard, 1970; Goldlust et al., 1974) results in the concomitant expression of two important biological activities resident in the C5a and C5b fragments (Müller-Eberhard, 1975): (1) the C5a-directed (a) degranulation of mast cells (Cochrane & Müller-Eberhard, 1968; Vallota & Müller-Eberhard, 1973; Johnson et al., 1975), (b) chemotaxis of polymorphonuclear leukocytes (Shin et al., 1968; Jensen et al., 1969; Ward & Newman, 1969; Vallota et al., 1973), and (c) contraction of smooth muscle (Shin et al., 1968; Cochrane & Müller-Eberhard, 1968; Vogt, 1968; Wissler, 1972); and (2) the assemblage of the C5b–9 cytolytic complex (Kolb et al., 1972; Mayer, 1972; Kolb & Müller-Eberhard, 1973; Podack et al., 1976) initiated by the capacity of nascent C5b to bind C6 and C7 (Lachmann & Thompson, 1970; Arroyave & Müller-Eberhard, 1973; Goldlust et al., 1974).

The C5 protein is comprised of two polypeptide chains, $\alpha$ and $\beta$, with respective molecular weights of about $1.4 \times 10^5$ and $8 \times 10^4$ (Nilsson & Mapes, 1973; Nilsson et al., 1975). In vitro cleavage studies of highly purified C5 with the $\overline{EAC4b,2a,3b}$ cell intermediate have established that the cationic C5a fragment is derived from the $\alpha$-polypeptide chain (Nilsson et al., 1975). Preparative methods have been developed for the generation and isolation of C5a from human and porcine serum (Vallota et al., 1973; Vallota & Müller-Eberhard, 1973). Recently, the preparative method for C5a isolation from human serum has been improved and the fragment characterized with respect to its amino acid and carbohydrate compositions, molecular weight (11 000), and amino terminal structure (Fernandez & Hugli, 1976).

In consideration of the important biological activities of the C5 molecule, specifically the C5a and C5b fragments derived from limited proteolysis, we have developed a preparative method of purification from human plasma that also provides for the copurification of C3 (Tack & Prahl, 1976) and C4 (Bolotin et al., 1977). Our goal has been to obtain each of these proteins in a native state, in high yield, and in a high degree of purity. We have therefore taken specific measures to suppress endogenous proteolytic enzymes throughout the purification and have avoided chromatographic and precipitin steps that would compromise recoveries or would be limited to small-scale application. The present report describes first the approach to purification and then the results of compositional and structural analyses of C5 and the constitutive polypeptide chains. A comparative analysis with the polypeptide chains of C3 will also be included.

## Materials and Methods

Plasma prepared from freshly drawn human blood collected in acid citrate–dextrose was obtained from the Washington Regional Blood Center of the American National Red Cross. EDTA,[1] $PhCH_2SO_2F$, L-lysine, Dip-F, Tris, 6-aminohexanoic

† From the Immunochemistry Section of the American National Red Cross Blood Research Laboratory, Bethesda, Maryland 20014, and the Department of Pathology, College of Medicine, University of Utah, Salt Lake City, Utah 84132. *Received September 13, 1978.* This work was supported in part by U.S. Public Health Service Research Grant AI 13843. A preliminary report of this work was presented at a meeting of the American Association of Immunologists in Anaheim, CA, May 1976.

* To whom correspondence should be addressed at the University of Utah.

‡ Present address: Laboratory of Chemical Biology, National Institutes of Health, Bethesda, MD 20014. Recipient of an Established Investigatorship (No. 77 168) from the American Heart Association.

[1] Abbreviations used: PEG, poly(ethylene glycol); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Dip-F, diisopropyl fluorophosphate; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; Pth, phenylthiohydantoin; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; glucose–$GVB^{2+}$ buffer, 2.4 mM barbituric acid buffer (pH 7.3) containing 2.5% glucose, 0.05% gelatin, 0.073 M NaCl, 0.15 mM $CaCl_2$, and 0.5 mM $MgCl_2$; $TosPheCH_2Cl$, L-1-tosylamido-2-phenylethyl chloromethyl ketone.

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acid, and proteins used as molecular weight markers in polyacrylamide gel electrophoresis were purchased from Sigma. Sepharose 6B, 4B, and CL-4B and protein A–Sepharose CL-4B were obtained from Pharmacia Chemicals. DEAE-cellulose (Whatman DE52) was purchased from Reeve Angel, and hydroxylapatite (Bio-Gel HT), polyacrylamide gel electrophoresis reagents, and AG2-X10 resin (chloride form) were from Bio-Rad Laboratories. Guanidine hydrochloride, 3 N mercaptoethanesulfonic acid, DTT, and sequenator reagents were products of Pierce Chemical Co. Flaked PEG 4000 and dialysis tubing were from Union Carbide. Diaflo ultrafiltration cells and membranes were purchased from Amicon. Functionally pure human complement components (C2, C3, C5, C6, C7), guinea pig complement components (C8 and C9), and the $EAC1_{gp}4_{hu}$ cell intermediate were obtained from Cordis Laboratories. Iodoacetamide and 2-mercaptoethanol were from Aldrich Chemical Co. Urea was purchased from J. T. Baker Chemical Co. and deionized as a 9 M solution by passage through a Barnstead mixed bed ion-exchange resin cartridge. Sodium dodecyl sulfate was a product of the British Drug House Ltd. Bovine $TosPheCH_2Cl$-trypsin, bovine carboxypeptidase A, and soybean trypsin inhibitor were obtained from Worthington Biochemical Corp. Yeast carboxypeptidase Y was a gift from Dr. Tony Hugli (Scripps Clinic and Research Foundation, La Jolla, CA).

*Immunochemical Techniques and Reagents.* Total C5 protein was quantitated by radial immunodiffusion as described by Mancini et al. (1965). The C5 immunodiffusion plates were purchased from Meloy Laboratories and developed according to the manufacturer's instructions. Immunoelectrophoresis in 1% agarose was performed according to Scheidegger (1955) in Tris–barbital/sodium barbital buffer (pH 8.8). Standard double-immunodiffusion analyses were carried out on agarose plates purchased from Hyland Laboratories. Goat anti-human C5 was obtained from Meloy Laboratories, and rabbit antisera to human IgG, IgA, C3, and C4 and plasminogen were purchased from Behringwerke. A guinea pig antiserum to the post-Sepharose C3/C5 pool was raised in our laboratory by standard procedures.

*Hemolytic Assays.* A quantitative assay of C5 hemolytic activity remaining at each stage of purification was performed using an $EAC1_{gp}4_{hu}$ cell intermediate and functionally pure human C2, C3, C6, and C7 and guinea pig C8 and C9. Specifically, $2 \times 10^7$ $EAC1_{gp}4_{hu}$ cells were incubated with 0.2 mL of a prealiquoted C2, C3, C6, C7 reagent, with respective hemolytic titers of 100, 50, 50, and 50 $CH_{50}$ units/mL, and 0.2 mL of a diluted sample (equivalent to 0.1 to 0.01 $\mu$L of unknown) for 30 min at 30 °C. Then 0.2 mL of a prealiquoted C8, C9 reagent was added, with respective hemolytic titers of 50 $CH_{50}$ units/mL, and a second incubation performed at 37 °C for 60 min. Cell lysis was stopped by the addition of 2.0 mL of ice-cold 0.15 M NaCl, each reaction mixture was centrifuged at 1800 rpm to remove cellular debris and unlysed cells, and the absorbance at 541 nm was measured with a Gilford Model 340-N spectrophotometer. The hemolytic unit was the reciprocal of the dilution of C5 that would lyse $1 \times 10^7$ cells. All hemolytic components were stored at −70 °C in glucose–$GVB^{2+}$ buffer.

*Polyacrylamide Gel Electrophoresis.* Polyacrylamide gel electrophoretic analyses of C5 preparations were carried out according to the method of Weber & Osborn (1969) in 0.05 M sodium phosphate buffer (pH 7.0), 0.1% in sodium dodecyl sulfate. Gels with an acrylamide monomer concentration of 5.9% and an acrylamide:bisacrylamide ratio of 87:1 were found to provide optimal resolution. Samples (1–2 mg of protein/mL) were prepared for electrophoresis by dialysis against 0.05 M sodium phosphate buffer (pH 7.0), 5 mM in EDTA, then diluted with an equal volume of 2% sodium dodecyl sulfate in the absence or presence of 2% 2-mercaptoethanol and placed in a boiling water bath for 2 min. Alternatively, samples were incubated at 37 °C for 2 h in lieu of boiling. *Escherichia coli* $\beta$-galactosidase, rabbit muscle phosphorylase, *Aspergillus niger* glucose oxidase, beef liver catalase, pig heart fumarase, and bovine pancreas $\alpha$-chymotrypsinogen were used as molecular weight markers of 130 000, 100 000, 75 000, 60 000, 49 000, and 25 700, respectively. Gels were stained for 2–4 h with a 0.25% solution of Coomassie brillant blue R-250, 45% in methanol and 9% in acetic acid. Gels were destained by transverse electrophoresis against 5% methanol and 7.5% acetic acid.

*Reduction and Alkylation of C5.* C5 samples were desalted by dialysis against 0.1 M acetic acid and lyophilized. The lyophylate was dissolved in 0.2 M Tris-HCl buffer (pH 8.2) containing 6 M guanidinium chloride, 5 mM EDTA, and 20 mM DTT to give a protein concentration of 6–10 mg/mL. After a 2–3-h incubation at 37 °C, iodoacetamide freshly dissolved in a minimal volume of water was added dropwise to a final concentration of 42 mM. During this time the pH was maintained between 7.2 and 8.2 by the addition of 1 N sodium hydroxide. The 37 °C incubation was continued for 30 min and the reduced and alkylated protein then desalted by dialysis against 0.1 M acetic acid.

*Isolation of C5 Polypeptide Chains.* C5 that had been reduced, alkylated, and desalted was dialyzed against 0.1 M ammonium bicarbonate (pH 7.9), 1% in sodium dodecyl sulfate, for a minimum of 24 h at room temperature. The sample was then made 10% in glycerol and, after a trace of bromophenol blue was added, density layered on a $2.5 \times 160$ cm column of Sepharose CL-4B equilibrated with 0.1 M ammonium bicarbonate (pH 7.9), 0.2% in sodium dodecyl sulfate.

*Removal of Bound $NaDodSO_4$ from C5 Polypeptide Chains.* The method of Lenard (1971) was used to quantitatively remove bound sodium dodecyl sulfate from the $\alpha$ and $\beta$ chains of C5. A lyophilized sodium dodecyl sulfate–protein sample was dissolved in a minimal amount of distilled water and dialyzed at room temperature against 0.1 M acetic acid, 6 M in deionized urea, to remove free and some bound sodium dodecyl sulfate. The sample, 10–40 mg of protein, was then applied to a $0.7 \times 15$ cm column of AG2-X10 resin equilibrated in the same solution. Urea was removed from the unretarded protein sample by dialysis against 0.1 M acetic acid and either the sample was lyophilized for amino acid analysis or the pH was increased to 5.6 or 8.9 for carboxypeptidase digestion.

*Amino Acid Analyses.* Amino acid analyses were performed on a JEOL 6-AH amino acid analyzer interfaced with an Autolab System AA computing integrator. All samples were reduced, alkylated, and desalted prior to hydrolysis with 2× glass-distilled, constant-boiling 6 N HCl at 110 °C in sealed glass ampoules evacuated to less than $5 \times 10^{-3}$ Torr. Tryptophan was determined by the method of Penke et al. (1974) with 3 N mercaptoethanesulfonic acid. Serine and threonine values were corrected for acid destruction by extrapolation to zero time and maximal values were taken for all other amino acids. Hydrolyses were performed in duplicate for 24, 48, and 72 h.

*Carboxypeptidase A and Y Digestions.* Carboxypeptidase A digestions were performed in 0.2 M sodium bicarbonate

Table I: Summary of C5 Purification

| | total C5[a] ($CH_{50}$ units) | yield (%) | total C5[b] (mg) | yield (%) | sp hemolytic act. ($CH_{50}$ units of C5/mg of C5) |
|---|---|---|---|---|---|
| (1) plasma | $8.3 \times 10^8$ | 100 | 227 | 100 | $3.7 \times 10^6$ |
| (2) 5–12% PEG precipitate | $6.6 \times 10^8$ | 80 | 164 | 72 | $4.0 \times 10^6$ |
| (3) post DE52 pool | $4.2 \times 10^8$ | 51 | 111 | 49 | $3.8 \times 10^6$ |
| (4) post-Sepharose 6B pool | $3.5 \times 10^8$ | 42 | 98 | 43 | $3.6 \times 10^6$ |
| (5) post Bio-Gel HT pool[c] | $2.7 \times 10^8$ | 32 | 65 | 29 | $4.2 \times 10^6$ |

[a] Determined by a specific hemolytic assay using the $EACl_{gp}4_{hu}$ cell intermediate and functionally pure C2, C3, and C6–C9 reagents. [b] Determined by radial immunodiffusion (Mancini et al., 1965). [c] Depleted of IgG, subclasses 1, 2, and 4, by adsorption onto a protein A–Sepharose column.

buffer (pH 8.9) at a substrate to enzyme ratio of 20:1 to 40:1 at 37 °C. Digestions were stopped by lowering the pH to 2.2 with 6 N HCl, norleucine was added as an internal standard, and the released amino acids were quantitated on the amino acid analyzer. Carboxypeptidase Y digestions were performed in 0.1 M sodium acetate buffer (pH 5.6) at a substrate to enzyme ratio of 50:1 to 240:1 at 37 °C.

*Sequence Analysis.* A JEOL Model 47K sequence analyzer was used for all sequence analyses employing a double-cleavage program with 0.25 M Quadrol. The protein, 50–150 nmol, was placed in the cup either in 0.1 M acetic acid or in 0.05% sodium dodecyl sulfate, 1 mM in *N*-ethylmorpholine, to improve the uniformity of the protein film and to increase solubility during the initial coupling steps. Pth-norleucine was placed in the fraction collector tubes as an internal standard. Thiazolinones were converted to Pth-amino acids and identified by several methods: back hydrolysis in sealed and evacuated ampoules with 5.6 N HCl, 0.1% in stannous chloride (Mendez & Lai, 1975); gas chromatography on SP400 (Pisano & Bronzert, 1969); and when necessary by high-pressure liquid chromatography (Zimmerman et al., 1973).

*Purification Procedure.* As C5 was observed to copurify with C3, it was isolated as a byproduct in the purification of human C3 as described earlier (Tack & Prahl, 1976). In summary, these steps included sequential PEG fractionation, plasminogen depletion, ion-exchange chromatography, and gel filtration. All data relevant to column dimensions, buffer components, and gradients used in these steps may be found in the earlier publication, and so will be omitted here to avoid duplication.

Resolution of C5 from C3 was accomplished by chromatography on hydroxylapatite (Tack & Prahl, 1976). The C5 pool was concentrated by ultrafiltration on an XM-50 membrane to 1.5 absorbance units (280 nm)/mL and dialyzed against 0.1 M sodium phosphate buffer (pH 7.0), 0.15 M in sodium chloride, 50 mM in $\epsilon$-amino caproic acid, and 5 mM in EDTA. Quantitative immunoprecipitant analyses revealed that this pool contained 20–40% IgG and less than 2% IgA. The IgG subclasses 1, 2, and 4 were removed by adsorption on to a column containing 1 g (lyophilized weight) of protein A–Sepharose CL-4B equilibrated in the above buffer. No attempt was made to remove the small IgA contamination. C5 obtained in this manner was stored at −70 °C and was the material used in all subsequent studies. Regeneration of the protein A–column was possible through washing with several column volumes of 1.0 M acetic acid.

## Results and Discussion

*Purification.* Quantitative immunoprecipitant and hemolytic assays were used to provide for two independent assessments of C5 recovery at each step in the purification, as summarized in Table I. A fraction of plasma $\beta$-globulins was obtained through precipitation with PEG 4000. The 5–12% PEG precipitate was dissolved in a volume equal to one-fifth of the original plasma volume and depleted of plasminogen by passage over a Sepharose 4B–L-lysine column. This fraction was shown to contain 80% of the total plasma C5 hemolytic activity and 72% of the C5 antigenic activity. Similarly high recoveries of C3 and C4 activities were also consistently observed in this fraction. Subsequent chromatography in DE52-cellulose and Sepharose 6B resulted in the separation of C3 and C5 from most other plasma proteins. On DE52-cellulose both proteins coeluted at a specific conductance of 6.5 $m\Omega^{-1}$ $cm^{-1}$ (4 °C) and again on Sepharose 6B at a position corresponding to 62% of the column bed volume. C4 was quantitatively removed on the DE52 column since this protein eluted later in the sodium chloride gradient at 7.5–8.0 $m\Omega^{-1}$ $cm^{-1}$ (4 °C). The post-Sepharose C3/C5 pool was quantitated by radical immunodiffusion to contain 85% C3, 9.5% C5, 4.2% IgG, and less than 0.2% IgA; 43% of both the original C5 hemolytic and antigenic activities were present in this pool.

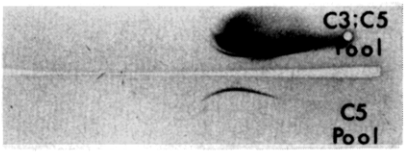


FIGURE 1: Immunoelectrophoretic analysis of the post-Sepharose 6B C3/C5 pool (upper well) and the post-hydroxylapatite C5 pool (lower well) developed with a guinea pig anti-human post-Sepharose C3/C5 antiserum.

The separation of C3 and C5 has usually been achieved by either stepwise or gradient phosphate elution from hydroxylapatite (Nilsson & Müller-Eberhard, 1965; Nilsson et al., 1972). The method used in the present work provides for the selective desorption of C5, IgG, and IgA from hydroxylapatite with 2 M potassium chloride (Hjertén, 1959) and the subsequent elution of C3 with 0.12 M potassium phosphate buffer, pH 7.4. Approximately 85% of the C5 hemolytic activity applied could be eluted with the 2 M potassium chloride. The post-hydroxylapatite C5 pool accounted for 33% of the total plasma C5 hemolytic activity and 29% of the antigenic activity. The specific activity of C5, expressed as $CH_{50}$ units/mg of C5 protein, remained high and constant at $\sim 4 \times 10^6$ throughout the purification, including the final adsorptive step on protein A–Sepharose.

*Immunochemical Analyses.* An antiserum raised in the guinea pig to the post-Sepharose 6B C3/C5 pool was multivalent as anticipated from prior functional and antigenic analysis of this pool (Tack & Prahl, 1976). Immunoelec-

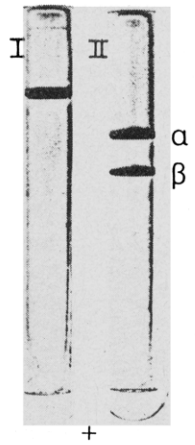


FIGURE 2: The polypeptide chain structure of post-hydroxylapatite C5 as shown by polyacrylamide gel electrophoresis in 0.1% sodium dodecyl sulfate (Weber & Osborn, 1969) in the absence and presence of mercaptoethanol. The samples shown are (gel I) nonreduced C5 and (gel II) mercaptoethanol-reduced C5.

trophoresis of the C3/C5 pool gave rise to two precipitin lines with this antiserum (Figure 1, upper well): a major anti-C3 line and a minor anti-C5 line of slightly greater anodal mobility. These assignments were confirmed with monospecific antisera to C3 and C5. The C5 pool from hydroxylapatite, however, gave only a single line with this antiserum (Figure 1, lower well) corresponding in position to the minor anti-C5 line of the starting material.

*Polyacrylamide Gel Electrophoresis.* Analysis of C5 by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate but in the absence of a reducing agent consistently gave a single band with a molecular weight of 190 000 ± 19 000 (Figure 2, gel I). When C5 was reduced prior to electrophoresis two polypeptide chains, designated $\alpha$ and $\beta$, were observed (Figure 2, gel II) with respective molecular weights of 115 000 ± 12 000 and 75 000 ± 8000, in reasonable agreement with the values of 141 000 ± 8500 and 83 000 ± 5000 reported by Nilsson et al. (1975). The apparent lack of dissociation in the absence of a reductive agent is taken to imply that the chain structure of this protein is stabilized in part through the presence of interchain disulfide bond(s). Collectively these data suggest that C5 purified in this manner retains a high specific hemolytic activity and approaches homogeneity within the limits of sensitivity of the functional, immunochemical, and electrophoretic analyses employed.

*Trypsin Digestion.* The susceptibility of C5 to digestion with bovine trypsin was assessed using polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate to detect changes in the structure of the molecule. C5 at a concentration of 1–2 mg/mL was digested with trypsin at a substrate:enzyme molar ratio of 13:1 (pH 7.4) for 0.5, 1.0, 10, and 30 min at 37 °C. At the indicated times a twofold excess of soybean trypsin inhibitor (w/w relative to trypsin) was added and one-half of the sample used directly for immunoelectrophoresis and the other half diluted with an equal volume of 2% sodium dodecyl sulfate with 2% 2-mercaptoethanol for gel electrophoresis. A preferential cleavage of the $\alpha$ chain was consistently observed with the parallel appearance of two major cleavage products (Figure 3a, gels A–E): a fragment, designated $\alpha_I$, of intermediate mobility relative to the $\alpha$ and $\beta$ chains of the control; and a fragment, $\alpha_{II}$, of considerably greater anodal mobility. The respective molecular weights of $\alpha_I$ and $\alpha_{II}$ were 89 000 ± 9000 and 28 000 ± 3000. The cleavage of the $\alpha$ chain to $\alpha_I$ and $\alpha_{II}$ was rapid and complete within 10 min (gels B–D). Digestion for an additional 20 min

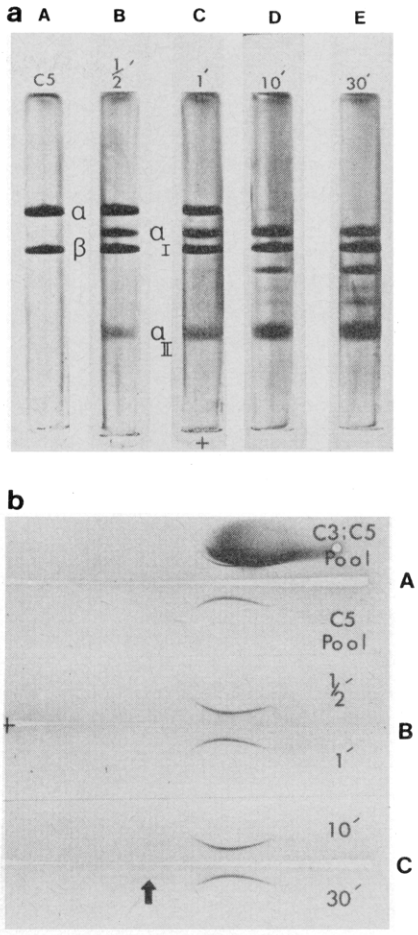


FIGURE 3: (a) Analyses of 1% (w/w) trypsin digests of native C5 by polyacrylamide gel electrophoresis in 0.1% sodium dodecyl sulfate. All samples were reduced with 2% mercaptoethanol prior to electrophoresis. The samples shown are (gel A) nondigested C5 and (gels B–E) 0.5-, 1.0-, 10-, and 30-min trypsin digests, respectively. (b) Immunoelectrophoretic analyses of trypsin digests of native C5. The samples shown are (plate A) the post-Sepharose C3/C5 pool (upper well) and post-hydroxylapatite C5 (lower well), (plate B) C5 digested for 0.5 (upper well) and 1.0 min (lower well), and (plate C) 10- (upper well) and 30-min digests (lower well). Each plate was developed with a guinea pig anti-human post-Sepharose C3/C5 antiserum.

(gel E) was without appreciable effect, although some intensification of a pre-$\beta$-chain fragment (molecular weight 60 000 ± 6000) was observed during this time period. The origin of this fragment was not apparent since a fragment of this size could result from cleavage of either the $\alpha_I$ fragment or the $\beta$ chain. The former alternative appears to be most consistent with our observations for the following reasons: (1) the intensity of stain in the $\beta$ region was clearly not diminished during the course of digestion; (2) cleavage of the $\beta$ chain to a fragment of 60 000 molecular weight would be expected to give a second fragment of about 15 000 molecular weight which was not observed during the above digestions; and (3) cleavage of $\alpha_I$ to a 60 000 molecular weight fragment would give a second fragment of approximately $\alpha_{II}$ size and, therefore, would likely remain undetected by gel electrophoresis. The isolation of $\alpha_I$, $\alpha_{II}$, and the pre-$\beta$-chain fragment with subsequent end-group analysis will be required to confirm our assignments. The method of isolation and amino-terminal structure of $\alpha_{II}$ will be discussed in a later section of this paper.

A similar pattern of digestion has been reported by Nilsson et al. (1975) for C5 using bovine trypsin (substrate:enzyme molar ratio of 6.5:1) at pH 7.0, 30 °C; i.e., the $\alpha$ chain was rapidly cleaved with the parallel appearance of two fragments, designated by them as inter-$\alpha$-$\beta$ III and fragment 1–4, with

respective molecular weights of 109 000 ± 6500 and 33 000 ± 2000. This conversion, which was complete within 15–30 min, was then followed by a cleavage of inter-$\alpha$-$\beta$ III to a pre-$\beta$ fragment of 61 000 ± 3000 molecular weight and cleavage of fragment 1–4 to peptides with molecular weights of roughly 11 000. Therefore our results and those of Nilsson et al. (1975) are in agreement concerning the fragmentation pattern of C5 on digestion with bovine trypsin as assessed by polyacrylamide gel electrophoresis under denaturing and reducing conditions. Minta & Mann (1977) have reported that trypsinization of C5 results in the release of a terminal $\alpha$-chain fragment with a molecular weight of 15 000 analogous to the C5a fragment released by the C5 convertase ($\overline{C4b,2a,3b}$). These results are clearly different from our observations and those of Nilsson et al. (1975) which have indicated that the preferred trypsin cleavage site is far more interior giving an $\alpha$-chain terminal fragment ($\alpha_{II}$ or fragment 1–4) of considerably greater molecular weight. The basis for this difference is not apparent, although the specific hemolytic activity of the C5 isolated by Minta & Mann (1977) was not reported. As we have reported here, the activity of C5 remained constant ($\sim 4 \times 10^6$ $CH_{50}$ units/mg of C5) throughout the isolation procedure. The possibility must remain, therefore, that changes in the protein structure unrelated to its antigenic reactivity could account for the difference in fragmentation patterns.

In contrast to the results of Cochrane & Müller-Eberhard (1968), Nilsson et al. (1975), and Minta & Mann (1977) we have not observed a significant change in immunoelectrophoretic mobility of C5 upon digestion with trypsin for time periods extending up to 30 min (Figure 3b, plates A–C). However, a faint precipitin line of increased anodal mobility was detected in the 10- and 30-min digests (plate C) at the position indicated by the arrow. These data are taken to imply either (1) that the $\alpha_{II}$ fragment is unable to dissociate from the $\alpha_I$–$\beta$ chain complex in the absence of reduction and/or denaturation or (2) that $\alpha_{II}$ on dissociation does not appreciably affect the net charge of the $\alpha_I$–$\beta$ chain complex and therefore no change in mobility is detected. The failure of $\alpha_{II}$ to dissociate in the presence of 1% sodium dodecyl sulfate on gel electrophoresis would suggest, however, that this fragment is disulfide bonded to $\alpha_I$ and/or the $\beta$ chain.

*Isolation of C5 Polypeptide Chains.* A preparative separation of the $\alpha$- and $\beta$-polypeptide chains of C5 was obtained by gel filtration following the total reduction and alkylation of the protein in 6 M guanidinium chloride at pH 8.2. Initially, chain separation was achieved on a 2.5 × 160 cm column of Sepharose 4B in 6 M guanidinium chloride, 0.1 M ammonium bicarbonate (pH 8.0). Although this method gave a satisfactory separation of the two chains, several practical problems arose: (1) on removal of the guanidinium chloride both chains precipitated and therefore proved difficult to work with in subsequent analyses; and (2) all fractions from this column were grossly contaminated with carbohydrate material derived from the Sepharose matrix due to the structure breaking effects of the guanidinium chloride. For these reasons we changed to a gel matrix of improved chemical and mechanical stability (Sepharose CL-4B) with 0.2% sodium dodecyl sulfate, 0.1 M ammonium bicarbonate (pH 7.9) as the eluant. Following reduction and alkylation, the guanidinium chloride was removed by dialysis against 0.1 M acetic acid before a final equilibration with 1% sodium dodecyl sulfate, 0.1 M ammonium bicarbonate (pH 7.9). The sample was then made 10% in glycerol and density layered on a 2.5 × 160 cm column of Sepharose CL-4B equilibrated in the above buffer. The flow rate was maintained at 4 mL/h with a peristaltic pump and

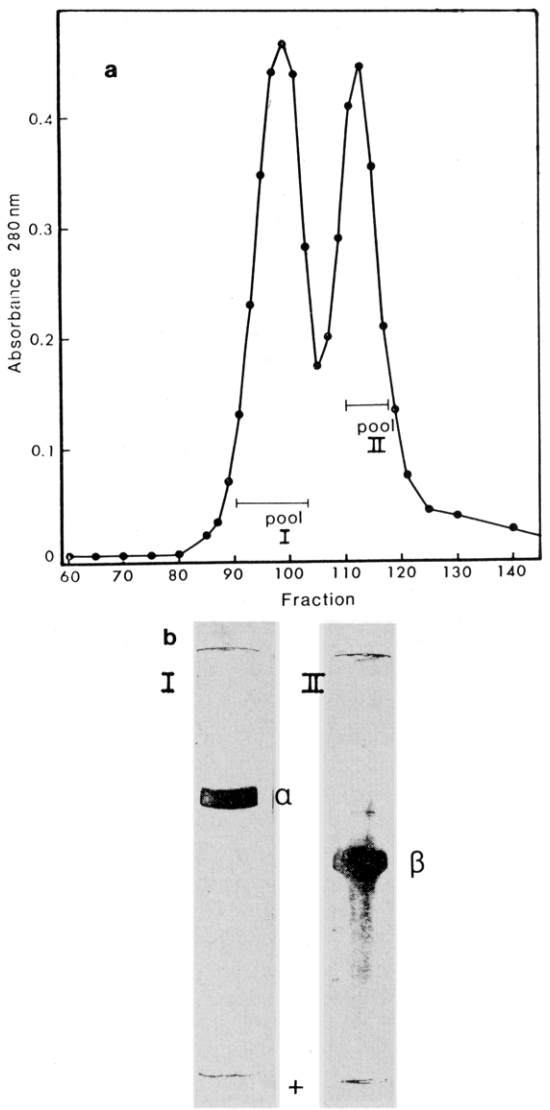


FIGURE 4: (a) Gel filtration of reduced and alkylated post-hydroxylapatite C5 on a 2.5 × 160 cm column of Sepharose CL-4B in 0.2% sodium dodecyl sulfate, 0.1 M in ammonium bicarbonate (pH 7.9). The absorbance at 280 nm is shown. Pools I and II were made as indicated. (b) Polyacrylamide gel electrophoresis in 0.1% sodium dodecyl sulfate of pools I and II (from a).

1.5-mL fractions were collected after 300 mL of buffer had eluted from the column. Two major protein peaks were observed on monitoring the effluent at 280 nm (Figure 4a). Pools I and II were made as indicated and subsequent polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Figure 4b; gels I and II) confirmed a complete separation of the $\alpha$ and $\beta$ chains. Each pool was concentrated by lyophilization and served as the source of material for all subsequent analyses.

*Amino Acid Analysis of C5 and C5 Chains.* The amino acid compositions of C5 and the constitutive $\alpha$ and $\beta$ chains of C5 are shown in Table II, expressed as moles of amino acid/mole of protein. These data were calculated on the basis of estimated molecular weights of 190 000, 115 000, and 75 000, respectively. The molecular weight of C5 was arrived at by the summation of the estimated weights of the $\alpha$ and $\beta$ chains, as the inherent error in estimating the weight of the native protein was considered greater than that of the constitutive chains. As we have not attempted to quantitate the carbohydrate content of C5 or its chains in these studies, no corrections were applied to the values given in Table II. In view of the reports of Müller-Eberhard (1969) that C5 contains

Table II: Amino Acid Compositions of Human C5 and the Constitutive Chains[a]

| | C5[b] | C5α[b] | C5β[b] | (C5α + C5β)/C5 |
|---|---|---|---|---|
| Lys | 116.3 | 75.8 | 43.0 | 1.02 |
| His | 31.6 | 20.8 | 11.3 | 1.02 |
| Arg | 55.6 | 37.8 | 15.1 | 0.95 |
| *S*-CMCys | 23.7 | 17.3 | 6.3 | 1.00 |
| Asp | 161.5 | 93.2 | 79.7 | 1.07 |
| Thr | 113.7 | 66.5 | 45.3 | 0.98 |
| Ser | 143.5 | 82.4 | 60.2 | 0.99 |
| Glu | 183.6 | 109.6 | 76.2 | 1.01 |
| Pro | 72.4 | 35.3 | 32.4 | 0.94 |
| Gly | 97.6 | 56.4 | 39.2 | 0.98 |
| Ala | 99.5 | 56.0 | 38.9 | 0.95 |
| Val | 123.9 | 75.2 | 52.8 | 1.03 |
| Met | 20.9 | 11.2 | 6.4 | 0.84 |
| Ile | 98.9 | 65.0 | 41.1 | 1.07 |
| Leu | 148.8 | 98.4 | 55.4 | 1.03 |
| Tyr | 79.6 | 45.2 | 37.2 | 1.04 |
| Phe | 68.9 | 40.5 | 29.5 | 1.02 |
| Trp | 9.5 | 7.2 | 1.7 | 0.94 |
| total residues | 1649 | 994 | 672 | 1.00 |

[a] Reported as moles of amino acid per mole of protein. [b] Calculated on the basis of molecular weights for C5, C5α, and C5β of 190 000, 115 000, and 75 000, respectively, uncorrected for the carbohydrate content.

15% carbohydrate and Fernandez & Hugli (1976) that the anaphylactic fragment of C5, i.e., C5a, contains 27% carbohydrate, these data must be evaluated with the appreciation of this shortcoming. As shown in Table II, the sum of the total number of residues calculated for each amino acid of the α + β chains divided by the corresponding number of residues independently determined for native C5 gave a quotient approaching 1.0 in most instances. This would argue that the carbohydrate distribution between the two chains is probably not highly discordant. Upon comparison, the α and β chains of C5 reveal significant dissimilarities in amino acid content. The α-chain content of lysine, histidine, arginine, cysteine, methionine, and tryptophan exceed that of the β chain by greater than 15 mol %, whereas the β-chain content of aspartic acid/asparagine, proline, and tyrosine exceed that of the α chain by the same degree.

It has been suggested on the basis of structural and functional similarities that C3 and C5 may have evolved from a common primordial gene (Müller-Eberhard, 1975; Prahl, 1976). A comparison of the compositions of C5 and its constitutive chains reveals marked differences which do not strongly support the contention of finding a profound structural homology at the level of the primary sequence. This conclusion must be tempered, however, by our paucity of knowledge concerning the structure of these large proteins. It is not clear, for example, if we are comparing chains of homologous length; neither do we know the genetic history of the proteins in terms of internal duplications or deletions nor are we aware of the possible processing of the nascent polypeptide chain(s). As will be discussed later, limited sequence data does support the concept of homology.

*Carboxyl-Terminal Analysis.* The carboxyl-terminal structures of the α and β chains of C5 have been determined using carboxypeptidases A (CpA) and Y (CpY). Initial efforts to obtain release of carboxyl-terminal amino acids with CpA in 0.1% sodium dodecyl sulfate at pH 8.9 or with CpY in 0.1% sodium dodecyl sulfate at pH 5.6 were unsuccessful. If bound sodium dodecyl sulfate was removed from the protein as described earlier and omitted from the digest buffer, a rapid release from both chains was seen with CpY and a significant but slower release with CpA. These results were interpreted to indicate a rapid loss of CpY activity in the presence of 0.1% sodium dodecyl sulfate. Although the relative stabilities of CpA and CpY in 0.1% sodium dodecyl sulfate have not been determined in these studies, a rapid release of alanine from reduced and alkylated C3 and from the C3 β chain has been observed with CpA in the presence of 0.1% sodium dodecyl sulfate (Tack & Prahl, 1976; Tack et al., 1979).

Digestion of the C5 α chain with CpY at a substrate to enzyme molar ratio of 120:1 in 0.1 M sodium acetate buffer (pH 5.6) for 60 min at 37 °C resulted in the release of 0.85 mol of Ser, 0.40 mol of Gly, 0.70 mol of Ala, and 0.49 mol of Val per mol of α chain. At a ratio of 240:1 we observed the release of 0.80 mol of Ser, 0.60 mol of Gly, 0.40 mol of Ala, and a trace amount of Val per mol of α chain after 10 min of digestion at 37 °C. A C-terminal sequence of (Ala,Val)-Ala-Gly-Ser was indicated from these data. Identical results were obtained on digestion with CpA, confirming the assigned sequence. Retrospectively, the lack of release with CpA in the presence of 0.1% sodium dodecyl sulfate was now interpretable; i.e., an X-Gly-Ser sequence is only slowly hydrolyzed by CpA and the denaturation of the enzyme, therefore, becomes a limiting factor under these conditions.

Digestion of the C5 β chain with CpY at a substrate to enzyme molar ratio of 50:1 (pH 5.6) for 10 min at 37 °C resulted in the release of 0.75 mol of Ser, 0.41 mol of Gly, 0.37 mol of Ala, and a trace amount of valine per mol of chain implying that C5 α and β chains share a common carboxyl-terminal structure. Since the retention time for serine on the long column of the amino acid analyzer in sodium citrate buffer (pH 3.2) is about the same as that for either asparagine or glutamine, both α and β chain digests were separately analyzed in a lithium citrate buffer (pH 2.8) capable of resolving serine, asparagine, and glutamine. The presence of serine was confirmed with this latter buffer system and identical stoichiometry observed.

*Amino-Terminal Studies.* A fifty-nanomole sample of native C5 was desalted by dialysis against 0.1 M acetic acid, lyophilized, and transferred to the cup of the sequenator in anhydrous trifluoroacetic acid. Ten cycles of Edman degradation resulted in the single amino-terminal sequence of Thr-Leu-Gln-Lys-Lys-Ile-Glx-Glx-Ile-Ala. Subsequent degradation of 40–60 nmol of C5 α chain prepared by dialysis against 1 mM *N*-ethylmorpholine containing 0.05% sodium dodecyl sulfate resulted in the identical sequence observed for the C5 protein. Calculated percent recoveries based on the Pth-norleucine internal standard are shown in Table III with the methods used for identification. Several attempts to sequence the isolated C5 β chain have been unsuccessful. This observation, coupled with the single sequence obtained with intact C5, suggests the amino terminus of this chain is blocked. However, the possibility remains that the amino terminus of the β chain has been rendered unsequenceable during handling by cyclization of an N-terminal glutamine to pyrrolidone carboxylic acid.

As described earlier, trypsin digestion of native C5 produces a rapid cleavage of the α chain into two fragments, i.e., $\alpha_I$ and $\alpha_{II}$, with respective molecular weights of 89 000 and 28 000. The $\alpha_{II}$ fragment was isolated from a 10-min trypsin digest of C5 (substrate to enzyme molar ratio of 13:1) following reduction and alkylation in 6 M guanidinium chloride by gel filtration on a 2.5 × 160 cm column of Sepharose CL-4B in 0.1 M ammonium bicarbonate (pH 7.9) containing 0.2% sodium dodecyl sulfate. The $\alpha_{II}$ fragment, which eluted after

Table III: Edman Degradation of Human C5 and C5 α Chain[a]

| | C5 | | | C5 α chain | | | |
|---|---|---|---|---|---|---|---|
| | | recovery | | | recovery | | |
| step | residue identified | nmol | % | residue identified | nmol | % | method of identification |
| 1 | Thr | 8.3 | 16.6 | Thr | 6.2 | 10.8 | HI |
| 2 | Leu | 9.3 | 18.5 | Leu | 14.3 | 25.1 | GC, HI |
| 3 | Gln | 6.8 | 13.5 | Gln | 6.5 | 11.4 | HI, LC |
| 4 | Lys | 4.6 | 9.1 | Lys | 6.6 | 11.5 | HI |
| 5 | Lys | 5.5 | 11.0 | Lys | 7.6 | 13.4 | HI |
| 6 | Ile | 7.5 | 15.0 | Ile | 6.6 | 11.6 | GC, HI |
| 7 | Glx | 8.8 | 17.5 | Glx | 6.6 | 11.6 | HI |
| 8 | Glx | 8.6 | 17.1 | Glx | 6.3 | 11.0 | HI |
| 9 | Ile | 7.0 | 14.0 | Ile | 5.7 | 10.0 | GC, HI |
| 10 | Ala | 10.8 | 21.5 | Ala | 6.6 | 11.6 | GC, HI |

[a] A sample of 9.5 mg (50 nmol) of intact C5 protein and 6.6 mg (57 nmol) of C5 α chain were loaded in the cup for the respective analyses. Present recovery is given based on the Pth-norleucine internal standard. Threonine was identified as α-aminobutyric acid on back hydrolysis. GC, gas chromatography; HI, back hydrolysis; LC, high pressure liquid chromatography.

the C5 β chain, appeared homogeneous on inspection by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. When subjected to automated Edman degradation for six cycles, a single sequence was observed, albeit in low yield, identical with that obtained with the isolated C5 α chain.

It would appear, then, that the initial cleavage of C5 by trypsin does not mimic the physiological activation of the protein (Nilsson et al., 1975), in contrast to the effect of this enzyme on C3 (Bokisch et al., 1969; Tack et al., 1979) since the resultant amino-terminal $\alpha_{II}$ fragment was appreciably larger than the C5a anaphylatoxin isolated by Fernandez & Hugli (1976). The $\alpha_{II}$ peptide should prove useful in establishing the sequence of amino acids at the physiological site of C5 activation.

The amino- and carboxyl-terminal studies have provided the following information: (1) the identity in amino-terminal structure (positions 1–10) between the C5 α chain and the C5a anaphylatoxin isolated from human sera (Fernandez & Hugli, 1976) provides direct evidence that this potent chemotactic and anaphylactic polypeptide is derived from the amino-terminal end of this chain; (2) the C5 β chain, either as present in native C5 or as purified by gel filtration in the presence of sodium dodecyl sulfate, has a blocked amino terminus since it was consistently resistant to Edman degradation; (3) the C5 α and C5 β chains appear to share a common carboxyl-terminal sequence, i.e., (Ala,Val)-Ala-Gly-Ser; (4) the C3 α and C5 α chains also have an identical carboxyl-terminal structure; and (5) the C3 β and C5 β chains are distinctive in their carboxyl termini with sequences of Pro-Ala-Ala and Val-Ala-Gly-Ser, respectively.

A recent report of Fernandez & Hugli (1977) reveals a striking homology when the first 25 residues of C5a and C3a are compared. Where only two residue positions are identical in the first 10 residues (or 20% homology), this increases to eight positions if the base is increased to 25 residues (or 33% homology). Despite the lack of support for common genic divergence by the compositional data, the increasing homology in the amino-terminal region and the identical carboxyl-terminal sequences observed in the α chains of C5 and C3 suggest further structural studies may support this concept. Evidence for homology of the β chains of C5 and C3 is, however, completely lacking at this time.

The entire primary sequence of the human C5a anaphylatoxin has recently been reported (Fernandez & Hugli, 1978). Although the C5a fragment is only 74 residues long, compared with 77 residues for the C3a fragment, it has been possible to align the sequences in such a manner as to maximize identities of 36% in the two sequences. The disulfide structure of six half-cystines in each molecule is also identical. As the authors point out, this relatedness is statistically significant, supporting the concept of a common genetic origin.

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# Third Component of Human Complement: Structural Analysis of the Polypeptide Chains of C3 and C3b†

Brian F. Tack,‡ Sam C. Morris, and James W. Prahl*

ABSTRACT: The $\alpha$- and $\beta$-polypeptide chains of human C3 have been isolated after total reduction and alkylation by gel filtration on agarose in the presence of sodium dodecyl sulfate. The amino acid and neutral sugar contents of the C3 protein and each chain are reported. Automated Edman degradation has established amino-terminal sequences for the $\alpha$ and $\beta$ chains of Ser-Val-Gln-Leu-Thr-Glu-Lys-Arg-Met-Asx-Lys-Val-Gly and Ser-Pro-Met-Tyr-Ser-Ile-Gly-Thr-Pro-Asx, respectively. Carboxypeptidase digestions have identified carboxyl-terminal sequences for the $\alpha$ and $\beta$ chains of (Ala,Val)-Gly-Ser and Pro-Ala-Ala. Proteolytic cleavage of the $\alpha$ chain, characteristic of C3 activation, was studied with trypsin. The $\alpha'$ chain of the resultant C3b fragment was also isolated after total reduction and alkylation by gel filtration. An amino-terminal sequence of Ser-Asn-Leu-Asp-Glu-Asp-Ile-Ile-Ala-Glu-Glu-Asp-Ile-Val was determined for this chain. The previous proposal concerning the assignment of the C3a anaphylactic peptide to the amino terminus of the C3 $\alpha$ chain [Budzko, D. B., et al. (1971) *Biochemistry 10*, 1166–1172] is substantiated by (1) the identity in the amino-terminal sequence of the C3 $\alpha$ chain and the C3a fragment [Hugli, T. E., et al. (1975) *J. Biol. Chem. 250*, 1472–1478], (2) the appearance of a unique amino terminal associated with the C3b $\alpha'$ chain, and (3) unchanged carboxyl-terminal sequences for the C3b $\alpha'$ and $\beta$ chains.

The third component of human complement (C3) participates in both the classical and alternative pathways of complement activation. Our present knowledge concerning the physicochemical properties of the native protein, its mode of activation, and specificity of interaction with particle surfaces can be summarized as follows: (1) the molecular weight of C3 has been estimated by Budzko et al. (1971) to be 185 000 using the gel electrophoresis method of Hedrick & Smith (1968) in agreement with the value of 187 650 ± 5650 determined by "low-speed" sedimentation equilibrium (Tack & Prahl, 1976); (2) polyacrylamide gel electrophoresis of C3 under denaturing and reducing conditions has shown that the protein is comprised of two polypeptide chains, $\alpha$ and $\beta$, with respective molecular weights of $1.1–1.4 \times 10^5$ and $7.5–8.0 \times 10^4$ (Nilsson et al., 1975; Molenaar et al., 1974; Bokisch et al., 1975; Tack & Prahl, 1976); (3) activation of C3 by the classical pathway C3 convertase, $\overline{C4b,2a}$, is effected by cleavage of a single peptide bond in the $\alpha$ chain releasing an activation peptide

† From the Immunochemistry Section of the American National Red Cross Blood Research Laboratory, Bethesda, Maryland 20014, and the Department of Pathology, College of Medicine, University of Utah, Salt Lake City, Utah 84132. *Received September 13, 1978.* This work was supported in part by U.S. Public Health Service Research Grant AI 13843. A preliminary report of these studies was presented at the Tenth International Congress of Biochemistry, Hamburg, West Germany, July 1976.

* To whom correspondence should be addressed at the University of Utah.

‡ Present address: Laboratory of Chemical Biology, National Institutes of Health, Bethesda, MD 20014. Recipient of an Established Investigatorship (No. 77 168) from the American Heart Association.

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