Large Scale Isolation of Functionally Active Components of the Human Complement System*

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In the present work a scheme is presented for the isolation of multiple components of human complement in a functionally and biochemically pure state and with full hemolytic activity. These preparative procedures allow high recovery of milligram and gram quantities of particular complement components from a large pool (2–11 liters) of fresh EDTA plasma in no more than four chromatographic steps. Many components (C3bINA, C5, C3, C1EI, C4, and C9) are recovered functionally pure or highly purified following the first chromatographic step employing DEAE-Sephacel and may be utilized as reagents with no further purification.

Prior to anion exchange, individual units of plasma are treated with inhibitors of complement activation and serum proteases, the pooled plasma is fractionated with polyethylene glycol, depleted of plasminogen on Sepharose-lysine, and rapidly ultrafiltered to low ionic strength and high protein concentration. The high degree of resolution of the components on DEAE-Sephacel subsequently obtained is demonstrated by the functional recovery and purification in a representative experiment as indicated (in their order of elution) for the following proteins: C3bINA (24%, 18-fold), C2 (74%, 12-fold), C7 (87%, 14-fold), factor B (55%, 8.7-fold), C8 (50%, 16-fold), C6 (82%, 25-fold), β1H (39%, 12-fold), C5 (62%, 111-fold), C3 (99%, 64-fold), C1EI (42%, 135-fold), C9 (80%, 297-fold), and C4 (78%, 164-fold). Other components separated by these procedures include C1q and C4 binding protein.

Additional steps described, which demonstrate the utility and effectiveness of this preparative scheme, have allowed isolation of C3, C5, and C7 as pure components with full hemolytic activity as judged by functional, immunochemical, and physicochemical criteria. C8, also isolated as a homogeneous protein, was recovered with partial hemolytic activity. All these components were recovered in high yield and in the purification as indicated: C3 (61%, 103-fold), C5 (24% 1350-fold), C7 (19%, 2260-fold), and C8 (32%, 547-fold). Complement components C6, β1H, factor B, and C2 in addition to C3bINA, C1EI, C4, and C9 are recovered partially purified with good activity and are amenable to further purification.

In pursuing work on the biological functions of complement, it became apparent that simpler and more efficient methods were required for preparation of milligram quantities of functionally active, biochemically pure, and well characterized components of the human complement system. Early experiments proved the feasibility of greatly simplified methods. The purpose of this paper is to present an approach for large scale purification of multiple complement components from a single pool of human plasma that meets these criteria.

It was shown that individual pools of inhibitor-treated human plasma (2–11 liters) can be processed to yield at least 14 complement components early in the procedure that are generally stable and amenable to further purification. The excellent resolution obtained by an initial DEAE-Sephacel ion exchange chromatographic step allowed the isolation of many complement components in a state of high functional purity that can be used directly as complement reagents. The utility of this procedure was established by the subsequent isolation of homogeneous and functionally pure components C3, C5, C7, and C8 in good yield with no more than three additional chromatographic steps for each protein.

In developing efficient procedures to maintain recovery and purity while limiting inactivation of individual complement components, we have taken measures first to eliminate activation of both the classical and alternative pathways and also to block intrinsic or potential proteolytic activities of human plasma during the purification. In our approach to the resolution and isolation of pure complement components, we have used the usual physicochemical and immunological parameters to assess purity but, following the approach of Nelson et al. (1) and Vroon et al. (2) we have used, as well, sensitive functional assays that allow the detection of contaminants far below the usual level of resolution.

MATERIALS AND METHODS

Complement Components and Assay Procedures

In this purification the chromatographic behavior of the various components was followed by functional hemolytic assays. For five components (C3bINA, factor B, C5, C3, and C4), antigenic assays

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1 The abbreviations used are: C3bINA, C3b inactivator; C1EI, C1 esterase inhibitor; symbols E, A, and C represent, respectively, a sheep erythrocyte, one molecule of IgM, or two molecules of IgG antibody, and complement components being designated by number, i.e. C1, C2, etc. The letters "a" and "b" are used to designate fragments of the complement molecules. EAC-7 is a shorthand designation for an intermediate lysible by C8 and C9; PMSF, phenylmethanesulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PEG, polyethylene glycol; mS, multiSiemen-conductance measurement where a Siemen is equivalent to a reciprocal ohm; EACA, ε-aminocaproic acid.
were performed as well. In most cases, standard functional assays were utilized.

For these assays guinea pig C1 and C9 were isolated as previously described (3, 4). Functionally pure guinea pig C5, C6, C7, and C8 were prepared as in Ref. 5. Guinea pig C2 and C3 were purchased from Cordis Laboratories, Miami, FL. The human components used in these assays or obtained as follows: C4, C2, C3, and C6 were purchased from Cordis Laboratories. Biochemically pure human C3 was prepared by the method of Tack and Prabh (6) with an added immunoadsorbent step to remove contaminating C5, IgG, and IgA. C5 was prepared following hydroxyapatite chromatography also, as in Ref. 6. Contaminating IgG and IgA were removed by immunochromatographic deletion. The human C5b6 complex was used in preparation of EAC-7 from EAC43h. This complex was prepared as in Ref. 7.

Methods of Functional Assay

Buffers were prepared as described by Hammer and et al. (7) except that buffer D** containing 0.20 M glucose. Human C2 was assayed by a modification of the assay of Borsos et al. (8) using EAC14 (9) prepared with guinea pig C1 and human C4. Human C4 was assayed by a minor modification of the assay of the method of Hammer et al. (10). Human C3, C5, C6, and C7 were titrated by modifications of the method of Nussensweig (New York University School of Medicine, New York, Scripps Clinic and Research Foundation, La Jolla, CA) and Victor Hans J. Muller-Eberhard (Department of Molecular Immunology, I1). goats housed at the NIH animal farm were immunized with the antigens listed below were obtained from the designated commercial reagent. ClEI was assayed by the method of Gigli (12). Human C3BINA and βH were assayed as in Ref. 13.

Antisera to Components

For analytical purposes, small volumes of the antisera to human proteins listed below were obtained from the designated commercial laboratories: goat anti-human serum and rabbit anti-fibrinogen (Cappel Laboratories, Downingtown, PA); goat anti-albumin, C4, and C5 (Meloy Laboratories, Springfield, VA); rabbit anti-IGG, α,-antitrypsin, α,-macroglobulin, α,-acid glycoprotein, plasminogen, C3, and Clq (Behring Diagnostics, Sommerville, NJ); rabbit anti-IgA and IgM (Calbiochem-Behring Corp., La Jolla, CA); goat anti-transferrin, α,-human serum-glycoprotein ceruloplasmin, P2-glycoprotein, C-reactive protein, C1q, C1r, C1s, C1 inhibitor, B and D, and factor D (Antibodies, Scarborough, ME); goat anti-C3BINA (Kent Laboratories, No. Vancouver, B.C.); goat anti-C1s (Kallestedt Laboratories, Chaska, MN).

Monospecific anti-C8 and anti-C4 binding protein were gifts of Drs. Hans J. Muller-Eberhard (Department of Molecular Immunology, Scripps Clinic and Research Foundation, La Jolla, CA) and Victor Nussensweig (New York University School of Medicine, New York, NY), respectively.

When large volumes of monospecific or polyclonal antisera were required (i.e. for immunoadsorbent preparation) sheep, hares, or goats housed at the NIH animal farm were immunized with the appropriate immungens. Fifty to 500 μg of protein were emulsified in 50% Freund's complete adjuvant (2 ml) and injected intradermally at sites along the animals' back.

Immunological Methods

Antigenic levels of selected proteins in plasma and purified fractions were quantified by radial immunodiffusion according to Mancini et al. (14). Immunoelectrophoresis was performed in 1% agarose (Sigma). Veronal-acetate buffer, pH 8.2, containing 0.03% sodium azide and 10 mM EDTA. Ouchterlony analyses of column fractions were carried out on diffusion plates prepared with phosphate-buffered saline containing 0.05% Na azide, 10 mM EDTA, and 1% agarose.

Antiserum Fractionation and Immunoadsorbent Preparation

A two-step procedure for using octanoic acid and DEAE-cellulose for the isolation of IgG from mammalian sera developed by Steinbuch and Audran (15) was employed to fractionate antisera and obtain pure IgG antibody. Antibody at 10 mg/ml was coupled to Sepharose 4B (Pharmacia) by the BrCN method of March et al. (16). Following coupling, the unreacted groups were masked with a solution of 0.1 M ethanolamine, pH 9.0. As a precaution against proteolytic degradation of the adsorbed material due to exposure to the immunoadsorbent, the Sepharose 4B-coupled IgG was treated with 1 mM PMSF for 30 min at 37 °C prior to use.

Polyacrylamide Gel Electrophoresis

Crued and purified protein preparations were examined by polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate. The monomer acrylamide concentration of the gel was 7.5%, with an acrylamide/bisacrylamide ratio of 37.5:1. Samples and standard molecular weight markers (Bio-Rad) were prepared and run concurrently under both reducing and nonreducing conditions. Details of the method are as described by Maizel (17).

Concentration and Buffer Equilibration of Protein

Small volumes of protein solutions (up to 1 liter) were concentrated and their buffer composition adjusted by using Amicon ultrafiltration cells with PM 30 membranes or by precipitation with PEG 4000 (J. T. Baker Chemical Co.) at levels determined to maximize recovery of the desired component(s) with subsequent solubilization in the appropriate buffer. Volumes of several hundred milliliters or less were concentrated in collodion bags (25,000- or 75,000-dalton exclusion). The processing of large volumes of protein solution (2-11 liters), in particular the preparation of plasma for chromatography on DEAE-Sepharose, was accomplished with a Pellicon Ultrafiltration System using 20 square feet of 30,000-dalton exclusion membrane, high volume heads, and pump (Millipore).

Purification Procedure

General Approach and Methodology

The purification scheme is presented in two sections. The first describes the preparation of human plasma for chromatography on DEAE-Sepharose and the chromatographic separation of the components. At this step a high degree of resolution of components was achieved with a very high yield of functionally active protein. For convenience, the column eluent was divided into eight distinct component-containing pools. The second section presents the further purification of complement components C3, C5, C7, and C8 obtained from the DEAE-Sepharose pools.

All procedures were performed at 4 °C unless otherwise indicated. Centrifugation of PEG-precipitated solutions was carried out at 14,000 g for 25 min in a Sorvall RC2B centrifuge. The conductivity of buffers and solutions was measured at 0 °C. All stock buffers and solutions were Millipore-filtered prior to dilution and use. The data presented, except where otherwise indicated, refer to the preparation of components from a 2-liter pool of plasma. In some experiments volumes of up to 11 liters of plasma were processed and these preparations were used where indicated as the source of several components which were purified further.

Part A: Preparation of Human Plasma for Chromatography on DEAE-Sepharose

Treatment of Plasma with Inhibitor Solutions—A volume of about 500 ml of platelet-free EDTA human plasma was obtained from each of four donors who was medicatin-free and had fasted prior to plasmapheresis. Each unit of fresh plasma was diluted while stirring with a buffered inhibitor solution containing 1 M KH2PO4, 0.2 M Na2EDTA, and 0.2 M benzamidine-HC1 that had been adjusted to pH 7.4 with NaOH (20 parts plasma to 1 part inhibitor). Following the addition of inhibitors the plasma was pooled and 0.1 M PMSF in anhydrous isopropl alcohol was added to a final concentration of 1 mM. The pH was adjusted to 7.4, if required.

Fractionation of Plasma with PEG 4000—The inhibitor-treated plasma was made 5% (w/v) in PEG 4000 by slow addition, with stirring, of the solid powder and allowed to equilibrate for 1 h. The precipitate which formed was removed by centrifugation, suspended in a 2-fold concentrated Sepharose-lysin buffer to be described, and stirred overnight at 4 °C. The 5% PEG supernatant containing the bulk of the plasma proteins and complement components was adjusted to a specific conductivity of 12 mS/cm by the addition of solid NaCl to plasmapheresis solution.

The solubilized precipitate was cleared by centrifugation at 14,000 g for 1 h. This solution containing C1q and C4 binding protein was filtered through glass wool, brought to 1 mM PMSF, and rapidly frozen into small 100-μl volume pellets by pumping into liquid nitrogen, and stored at -65 °C.

Plasminogen Depletion of the 5% PEG Supernatant—Sepharose 4B-L-lysine adsorbent was prepared by coupling 200 g of L-lysine/liter
of BrCN-activated Sepharose 4B. About 1.1 liters of this biospecific adsorbent were packed into a glass column 15 cm in diameter. The plasminogen capacity of the Sepharose-lysine adsorbent prepared as described was sufficient to deplete up to 3.5 liters of plasma/liter of adsorbent. A 50 mM K/Na phosphate buffer (pH 7.4, 11.8 mM/cm) containing 10 mM EDTA and 150 mM NaCl was used to equilibrate the column. The 5% PEG supernatant was applied and washed at a flow rate of 1300 ml/h. Residual nonspecifically bound protein was removed from the column by washing with a 2-fold concentrated buffer and included in the adsorbed plasma pool.

Following several column washes with the 2-fold concentrated buffer, the bound plasminogen was eluted with the concentration of the 5% PEG supernatant. The C3 preparation was applied to a column (10 liter; 8 cm) containing Sepharose CL-6B equilibrated with a 3.2 mM K/Na phosphate buffer (pH 7.4, 1.37 mM/cm) containing 6.7 mM benzamidine-HCl, and 31.8 mM EDTA, 6.4 mM benzamidine-HCl, and 33.3 mM EACA was prepared and prior to use was made 1 mM in PMSF and warmed to 15 °C. The plasminogen-depleted PEG supernatant was rapidly reduced in tonic strength by repeated sequential additions (4 times) of the 11.8 mM/cm diluent to a total volume of 4 liters and subsequent concentration of the adjusted pool to 2 liters using a Pellicon Ultrafiltration System at an average rate of 5 liters/h. The adjusted pool was then applied to a Sepharose CL-GB (Pharmacia), equilibrated with the plasminogen-depleted 5% PEG supernatant and stored frozen at −80 °C. The adsorbent was regenerated by sequential treatment with 10 mM NaOH and 10 mM phosphoric acid. After washing with starting buffer, the gel was stored in the cold in the presence of 0.02% azide.

Concentration and Ionic Strength Adjustment of the Plasma- 

den Plasminogen 


generated PEG Supernatant—The post Sepharose CL-GB plasma pool (13.2 mM/cm) was made 33 mM in EACA by the addition of solid inhibitor and allowed to warm to about 15 °C. A diluent solution of pH 7.4, 1.1 mM/cm, and containing 6.7 mM EDTA, 6.7 mM benzamidine-HCl, and 33.3 mM EACA was prepared and prior to use was made 1 mM in FBS and warmed to 15 °C. The plasminogen-depleted PEG supernatant was rapidly reduced in tonic strength by repeated sequential additions (4 times) of the 11.8 mM/cm diluent to a total volume of 4 liters and subsequent concentration of the adjusted pool to 2 liters using a Pellicon Ultrafiltration System at an average rate of 5 liters/h. The adjusted pool was then applied to a Sepharose CL-GB (Pharmacia), equilibrated with the plasminogen-depleted 5% PEG supernatant and stored frozen at −80 °C. The adsorbent was regenerated by sequential treatment with 10 mM NaOH and 10 mM phosphoric acid. After washing with starting buffer, the gel was stored in the cold in the presence of 0.02% azide.

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of Sephadex G-200 equilibrated with the same buffer. Fractions of 20 ml were screened for β1H antigen and also for C6 and C8 functional activities. Two pools were made, one containing β1H and the other C6 and C8 activities, and were adjusted to 16% PEG (w/v) by addition of the solid and stirred for 1 h at 0 °C. The precipitates were collected by centrifugation. The β1H precipitate was solubilized in the above phosphate buffer at pH 7.0. The precipitate containing the C6 and C8 protein was suspended in a small volume of 4 M NaCl solution and subsequently dissolved by dilution with a pH 6.0, 5 mM Na phosphate buffer containing 100 mM NaCl and 0.005% gelatin. The conductance of this solution was then adjusted to 2.6 mS/cm by the addition of distilled water.

C8: CM-cellulose Chromatography—The concentrated C6- and C8-containing post-Sephadex G-200 pool was applied to a column (1.6 x 95 cm) of CM-cellulose equilibrated with a pH 6.0, 5 mM Na phosphate buffer containing 40 mM NaCl and 0.005% gelatin (2.6 mS/cm). The column was washed with 200 ml of starting buffer at 30 ml/h, collecting 12-ml fractions. The C6 and C8 proteins were eluted by application of a 580-ml linear salt gradient, the limit buffer containing 270 mM NaCl (14.7 mS/cm). The C6- and C8-containing fractions were pooled separately.

RESULTS

Part A: Preparation of the Plasma for DEAE-Sephadex Chromatography

Pooled plasma adjusted with PEG to a final concentration of 5% (w/v) lost all C4 functional activity and more than 90% of the C2 activity. It was critical to mix individual units of plasma with EDTA and benzamidine at final concentrations of 10 mM each before they were pooled, adjusted to 1 mM PMSF, and fractionated with PEG to yield a stabilized supernatant solution which contained at least 12 complement components of interest. As shown in Table I, Step A2, this latter procedure allowed recovery of 10 complement components in the 5% PEG supernatant with a functional yield of 95% or more. The only exception was C5, in which 83% of the functional activity was retained. Factor B antigen was present at 97% of the level in the original 2-liter plasma pool.

Plasminogen depletion of plasma reported (6, 18, 19) to be necessary for the subsequent good recovery of C3 and C5 could be achieved using 1 liter of Sepharose-lysine biospecific adsorbent/3.5 liters of plasma. One liter of adsorbent/2 liters of plasma was used here to insure complete removal of plasminogen. No plasminogen could be detected by double diffusion analysis in the concentrated post-Sepharose-lysine pool (88.8 A280/ml).

In order to obtain a concentrated soluble pool of low ionic strength for application to the DEAE chromatographic column, it was critical to use the Pellicon Ultrafiltration System as described earlier. If direct rapid decrease in volume of the complement pool was attempted, the concentration of residual PEG led to precipitation of protein and loss of components, especially C5 and β1H. The post-concentration pool contained 69% of the initial plasma protein, while only three complement components, β1H, C5, and C7, were reduced to this level of functional activity (Table I, Step A3). All other components lost no or little activity when maintained under the conditions of low ionic strength (1.5 mS/cm) and high protein concentration (89 A280/ml) required for subsequent DEAE-Sephadex chromatography.

DEAE-Sephadex Chromatography—Although it is known that DEAE-52 (Whatman) cellulose chromatography of guinea pig serum will resolve functional C3 and C5, 5 this is not so when human serum is substituted (6). Initial experiments utilizing DEAE-Sephadex, however, demonstrated that human C5 could be readily separated from human C3 and other complement components. In addition, other complement factors (C3bINA, C4, and C9) were also individually resolved. When the adjusted post-Sepharose-lysine pool was chromatographed on DEAE-Sephadex and the elution position of all the complement components identified by antigenic and/or functional assay, the results shown in Fig. 1 were obtained. In addition to C3bINA eluting at 1.35 mS/cm, C5 (5.5 mS/cm), C9 (10.0 mS/cm), and C4 (11.6 mS/cm) were individually resolved and pooled as such. While C2 (1.7 mS/cm) was almost completely separated from C7 and factor B (co-migrating at 2.2 mS/cm), these three components were pooled together to obtain maximal recovery. C8 (2.6 mS/cm), C6 (2.8 mS/cm), and β1H (3.4 mS/cm) overlapped sufficiently with each other so that one pool of these three components was made. In some experiments the elution position of C6 varied such that it would co-chromatograph with β1H rather than C8. C3 (6.6 mS/cm) was effectively separated from C5 as expected, but overlapped with about 30% of the C1EI (7.4 mS/cm), and they were pooled together (C3, C1EI-1). A pool of C1EI (C1EI-2) was made of the remainder of the C1EI (57%) that co-chromatographed with ceruloplasmin (7.8 mS/cm).

A summary of the purification of Part A, including stepwise recovery of protein and functional activity for each component, is presented in Table I. The C3bINA, the C1EI from C3 (C1EI-1), C1EI-2, C9, and C4 pools were adjusted with the solid PEG as indicated in Table I, Step A5, and the respective components were recovered and solubilized. A comparison of the amount of functional activity that was present in each of the dilute pools (not shown) with that obtained following concentration with PEG demonstrated, with the exception of factor B, the effectiveness in using appropriate concentrations of PEG for rapid and quantitative recovery of the components. It was found by radial immunodiffusion analysis that approximately half of the factor B antigen was not precipitable by PEG at 20% (w/v), the concentration found optimal for recovery of C7. The functional contamination of components isolated following DEAE-Sephadex chromatography, but purified no further, is shown in Table II. The lack of significant functional cross-contamination with other complement components for the single component pools of C3bINA, C1EI, C9, and C4 clearly demonstrated the high degree of resolution obtained following this first chromatographic step.

Part B: Isolation of Pure Components

Data for complete quantitation of each component at each step of the purification in Part A are shown for a single 2-liter plasma pool. Because complete quantitation of each component in Part B was not available for this 2-liter purification, the quantitation of components in typical pools of other preparations is shown.

Isolation of C3—The data for recovery of protein and C3 functional activity following each step are given in Table III. 5 The second 16% PEG precipitation shown in Step A5 was effective in removing 5% of the protein including trace amounts of ceruloplasmin and was not required if care was taken to exclude this protein when making the C3-containing pool following DEAE-Sephadex chromatography. Gel filtration of the C3 on Sepharose CL-6B removed a small amount of high molecular weight contamination as shown in Fig. 2. Functional and antigenic analysis of this C3 preparation revealed contamination with 4,200 units/ml of C5, 0.24 mg/ml of IgG, and a trace of IgA. These contaminations were com-

5 Portions of this paper (including Figs. 2 to 9 and Tables III to VI) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20014. Request Document No. 80M-1758, cite author(s), and enclose a check or money order for $5.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
**Table I**

*Summary of complement purification (Part A)*

Complement components are listed in their order of elution from the DEAE-Sephacel column (left to right) and multiple component pools prepared by PEG precipitation (Step A5) are indicated by brackets.

<table>
<thead>
<tr>
<th>Component Pools</th>
<th>CibINA</th>
<th>C2</th>
<th>C7</th>
<th>B*</th>
<th>C8</th>
<th>C6</th>
<th>/H</th>
<th>C5</th>
<th>C3</th>
<th>C1E1</th>
<th>C9</th>
<th>C4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step A1: Pooled human plasma (1975 ml, A_{280}/ml = 60.0 (100% yield))</td>
<td>5.29</td>
<td>2.82</td>
<td>101</td>
<td>122</td>
<td>121</td>
<td>49.0</td>
<td>3.40</td>
<td>101</td>
<td>22.8</td>
<td>18.7</td>
<td>95.0</td>
<td>129</td>
</tr>
<tr>
<td>Activity (× 10^3 units/ml)</td>
<td>87.8</td>
<td>46.8</td>
<td>1670</td>
<td>2.03</td>
<td>2010</td>
<td>813</td>
<td>56.4</td>
<td>1680</td>
<td>378</td>
<td>310</td>
<td>1570</td>
<td>2140</td>
</tr>
<tr>
<td>Specific activity (units/A_{280})</td>
<td>4.64</td>
<td>3.04</td>
<td>88.4</td>
<td>108</td>
<td>107</td>
<td>59.0</td>
<td>3.30</td>
<td>77.2</td>
<td>21.8</td>
<td>28.7</td>
<td>91.0</td>
<td>123</td>
</tr>
<tr>
<td>Step A2: 5% PEG supernatant (2150 ml, A_{280}/ml = 46.8 (85% yield))</td>
<td>98.8</td>
<td>64.8</td>
<td>1880</td>
<td>2.31</td>
<td>2280</td>
<td>1260</td>
<td>70.9</td>
<td>1640</td>
<td>464</td>
<td>611</td>
<td>1940</td>
<td>2610</td>
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<tr>
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<td>117</td>
<td>95</td>
<td>97</td>
<td>96</td>
<td>131</td>
<td>107</td>
<td>83</td>
<td>104</td>
<td>167</td>
<td>105</td>
<td>104</td>
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<tr>
<td>Recovery of activity (%)</td>
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<td>1.91</td>
<td>66.2</td>
<td>75.0</td>
<td>62.7</td>
<td>31.0</td>
<td>1.76</td>
<td>54.1</td>
<td>14.3</td>
<td>16.8</td>
<td>58.0</td>
<td>83.0</td>
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<td>2310</td>
<td>2.62</td>
<td>2190</td>
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<td>61.5</td>
<td>1890</td>
<td>500</td>
<td>587</td>
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<td>2900</td>
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<td>Specific activity</td>
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<td>108</td>
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<td>103</td>
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<tr>
<td>Recovery of activity (%)</td>
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<td>6.36</td>
<td>150</td>
<td>258</td>
<td>204</td>
<td>98.0</td>
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<td>47.3</td>
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<td>276</td>
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<td>Step A3: Sepharose-lysine (3160 ml, A_{280}/ml = 28.6 (76% yield))</td>
<td>104</td>
<td>71.6</td>
<td>1690</td>
<td>2.91</td>
<td>2300</td>
<td>1100</td>
<td>52</td>
<td>167</td>
<td>462</td>
<td>533</td>
<td>1770</td>
<td>3110</td>
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<tr>
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<td>69</td>
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<td>93</td>
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<td>84</td>
<td>117</td>
<td>77</td>
<td>99</td>
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<tr>
<td>Recovery of activity (%)</td>
<td>45</td>
<td>20</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>16-45</td>
<td>Pool 1</td>
<td>Pool 2</td>
<td>45</td>
<td>20</td>
<td>16-45</td>
<td>Pool 1</td>
</tr>
</tbody>
</table>

| Volume | 80.0 | 132 | 120 | 115 | 130 | 150 | 120 | 130 | 150 | 93.0 |
| A_{280}/ml | 19.0 | 56.8 | 31.9 | 5.80 | 14.2 | 5.36 | 8.20 | 2.14 | 6.07 |
| Recovery of A_{280} (%) | 1.3 | 6.3 | 3.2 | 0.56 | 1.6 | 0.29 | 0.9 | 0.27 | 0.47 |
| Activity (× 10^3 units/ml) | 30.0 | 31.3 | 1310 | 992 | 990 | 658 | 22.1 | 1080 | 343 | 156 | 41.2 | 998 | 2130 |
| Recovery of activity (%) | 24 | 24 | 97 | 87 | 54.6 | 50 | 82 | 39 | 62 | 99 | 27 | 15 | 80 | 78 |
| Specific activity (× 10^3) | 1.62 | 0.551 | 23.1 | 17.5 | 31.1 | 20.6 | 0.692 | 186 | 24.2 | 29.1 | 5.03 | 467 | 351 |
| Purification (fold) | 18.4 | 11.8 | 13.8 | 8.6 | 15.5 | 23.3 | 12.3 | 111 | 63.9 | 93.9 | 162 | 297 | 164 |

*Factor B (FB) antigen (nanograms/ml) determined by single radial immunodiffusion (14).*
Isolation of Human Complement Components

and resulted in a further purification of 2.3-fold. Recovery of C5 was 24% with an overall purification of 1350-fold (Table IV, Step B5).

Isolation of C7—When C7 was isolated from a pool of fractions obtained following DEAE-Sepharose chromatography devoid of most C2 activity, purification to homogeneity was achieved in three sequential chromatographic steps (Table V, Steps B6, 7, and 8). With respect to C7, the C2-deficient C7 pool was 18-fold purified over plasma; treatment with PEG prior to further chromatography removed 64% of the A_500 but little C7 functional activity (Table V, Step A5b). Chromatography of the C7 preparation a second time on DEAE-Sepharose shown in Fig. 5 and subsequently on CM-cellulose shown in Fig. 6 gave a product almost pure as determined by SDS-PAGE (not shown). The only significant contamination detectable by Coomassie blue protein stain was a pair of bands just entering the gel. Both these high molecular weight components reduced to a pair of diffuse bands when electrophoresed in the presence of mercaptoethanol, one of which co-migrated with albumin. Chromatography of C7 on DEAE-Sepharose was very efficient with respect to recovery of functional activity; chromatography using CM-cellulose, however, resulted in a substantial loss of activity (Table V, Step B7). The high molecular weight contaminants in the C7 preparation were effectively removed by gel filtration on Sepharose CL-6B as shown in Fig. 7. The final C7 product was 2260-fold purified over the pooled plasma with a recovery of 19%. Trace contamination of this preparation with IgG and IgA could be removed by selective immunoabsorption on Sepharose-coupled anti-IgG and anti-IgA.

Isolation of C8—The DEAE-Sepharose chromatography pool containing C8, C6, and β1H was used as the source of C8 for purification. The quantitative data (Table VI) of protein and functional recovery are presented for both C8 and C6; while C6 requires additional purification to obtain homogeneous protein, the C6 product was recovered in good yield and in a functionally clean state. In order to separate β1H and high molecular weight protein from C6 and C8, advantage was taken of the property of β1H to elute from Sephadex G-200 as a 300,000 dalton component (20). As shown in Fig. 8, β1H antigen was detected early in the elution profile with the bulk of the protein and well resolved from functional C6 and C8

...
activities. Separation of β1H from C6 and C8 was not attain-
able if the gel filtration was performed in low ionic strength
buffer (2.6 mS/cm) conditions to be used in the following step,
CM-cellulose chromatography. Under these circumstances C8
was found to aggregate and consequently was filtered early
resulting in a broad elution profile that overlapped both β1H
and C6. This C8 aggregation was found to be reversible if the
aggregated C8 was subsequently refiltered under conditions of
normal ionic strength. The recovery of C6 and C8 functional
activity from the gel filtration step was complete and resulted
in a 10-fold increase in purity for both components (Table VI,
Step B9). The following step, chromatographic separation on
CM-cellulose, resulted in an additional 2-fold purification for
C6 and 3.3-fold purification for C8 (Fig. 9). Based in part on
SDS-PAGE patterns of these purified proteins, it was appar-
ent that the C6 preparation still contained significant contain-
ing noncomplement protein. This C6 preparation was 502-
fold purified over plasma at this stage (Table VI, Step B10).
However, the C8 preparation purified 547-fold over plasma
when analyzed by SDS-PAGE was effectively pure as judged
by Coomassie blue staining (Fig. 10). The final yield of C8 and
C6 functional activity was 32% and 39%, respectively.

Homogeneity and Physicochemical Characterization of
the Purified Proteins—The purified preparations of C3, C5,
C6, and C8 revealed the polypeptide chain structures as shown
in Fig. 10 a, b, c, and d, respectively, when subjected to SDS-
PAGE analysis. Under reducing conditions C3 and C5 re-
vealed subunit structures consisting of two chains each, α
chains, estimated Mr = 127,000 and 123,000, and β chains,
estimated Mr = 75,000 and 80,000, respectively, in agreement
with previously reported values (6, 21, 22). C7 demonstrated
a single polypeptide chain of 103,000 daltons similar to that
reported by Podack et al. (23). Under nonreducing conditions,
C8 showed two subunits of 99,000 (α-γ) and 70,000 (β) daltons.
Reduction of C8 with mercaptoethanol (not shown) produced
a three-chain pattern upon analysis compatible with the re-
ported structure of this component (24).

Functional Purity of the Complement Components—The
C3 (9.9 × 10^4 units/ml), C5 (1.3 × 10^4 units/ml), C6 (1.5 × 10^5
units/ml), C7 (7.3 × 10^5 units/ml), and C8 (1.5 × 10^5 units/ml)
preparations were assayed for contamination by other complement
components. The results of hemolytic tests shown in Table VII demonstrate the high degree of functional
purity obtained for these proteins when analyzed at concen-
trations up to 100 times that found in plasma.

Immunochromatography Analysis—Ouchterlony analysis of the
purified components was performed at the following concen-
trations: C3, 3.3; C5, 1.5; C7, 1.7, and C8, 2.2 mg/ml. Of the 25
monospecific antisera indicated under “Materials and Meth-
ods” used to test for contamination, none reacted with the
homogeneous preparations of C3 or C5. The C7 preparation
reacted only weakly with anti-factor B and gave a trace
response to anti-IgG and IgA. The C8 preparation produced
a trace response only to anti-β1H of all antisera tested.

DISCUSSION

In this report we describe a preparative schema for the
isolation of multiple components of complement from one
large pool of human plasma in which the purified products
are characterized functionally, physicochemically, and immu-
nochemically. The fine resolution and degree of purity of
complement components obtained in the first chromatogra-
phic step utilizing DEAE-Sephacel ion exchange cellulose
are shown in Fig. 1. The distribution of complement compo-
nents as identified by functional activity and antigenic analysis
has been greatly improved in comparison to the resolution
obtained in multicomponent preparations reported by others
(1, 2).

It has been shown by several investigators that human C3
and C5 do not resolve well by chromatography on certain
types of weak anion exchange cellulososes (2, 6, 19, 25, 26),
presumably a result of their similar molecular structures (22).
It has been reported that human C6 and C7 which have very
similar physicochemical properties are consequently also dif-
ficult to resolve using anion exchange chromatography (23, 27,
28). The resolution of these four components, however, is
possible when DEAE-Sephacel is utilized as described here
(Fig. 1). Furthermore, the C5 and C3 post-DEAE preparations
were determined to be 20% and 86% pure, respectively. The
biochemical purity of the post-DEAE C4 preparation was
estimated to be >90%, as judged by SDS-PAGE (not shown).
A three-chain polypeptide structure was identified as 97,000,
72,000, and 36,000 daltons, respectively, for the α, β, and γ
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chains in agreement with others (29, 30). SDS-PAGE analysis of the post-DEAE C9 preparation (not shown) indicated a single, major polypeptide chain of 64,000 and better than 50% purity as judged by Coomassie blue staining.

To establish the feasibility of further purifying the component pools obtained from the DEAE-Sephacel step, we proceeded with component components C3, C5, C7, and C8. There are two previous reports that describe methods for the isolation of multiple components of complement (1, 2). Nelson et al. (1) in 1966 were the first to outline methods for the preparation of individual complement component pools from guinea pig serum. Later, using similar techniques, Vroon et al. (2), with somewhat larger volumes of human serum (200 ml), also reported methods for isolating functionally pure components from a single pool of human serum. The purity of the individual component preparations was assessed only by functional hemolytic assays; no attempt was made to establish the biochemical purity of their preparations. Further, these reports did not provide quantitative information regarding each component that would allow for characterization of the purified material as well as a comparison of the component quality for repeat preparations.

Although methods are available for the preparation of one or at most two pure complement components in fair yield from a single pool of serum, most of these original protocols prepared no data on functional purity and only partial functional activity is retained by the purified protein. There are no protocols for the preparation of multiple components with high resolution from a single pool of serum or plasma.

Thus, C3 isolated from pooled human plasma as described here is obtained with a 103-fold purification as a homogeneous protein (Fig. 10a). The product was recovered with a 1.6-fold gain in specific-hemolytic activity, accounting for 61% of the activity in plasma (Table III, Step B2).

The procedures used to purify human C5 (Table IV) are similar to those presented in Ref. 21. We found that to maintain C5 as well as β1H solubility and obtain good protein recovery, the adjustment of the plasminogen-depleted plasma pool to low ionic strength for chromatography on DEAE-Sephacel required the use of the Pellicon Ultrafiltration System as described earlier. The low ionic strength (1.35 mS/cm) for the pre-DEAE plasma pool was required for resolution of components eluting earlier than C5, as shown in Fig. 1. The immunochemically contaminant-depleted C5 product was shown to be a homogeneous protein by SDS-PAGE (Fig. 10b) with 24% of the functional activity present in plasma (Table IV, Step B5). This C5 preparation was 1350-fold purified as calculated by the increase in specific activity and represents a 1.5-fold gain in specific functional activity over that present in plasma. Human C5 was first isolated by Nilsson and Müller-Eberhard in 1965 (25) and later by Nilsson et al. (19) in an improved three-step procedure.

A method for the isolation of highly purified human C7 was first developed in 1973 (31). The product was reported, however, to retain little of its original hemolytic activity (23). Nilsson (28) reported a procedure that allowed separation of human C6, C7, and C8, but these products were not assessed as by physicochemical and immunological criteria. Recently, Podack et al. (23, 27) have described methods for purification of C6 and C7 from outdated human serum. Our procedure for purification of C7 requires four chromatographic steps if care is taken to exclude most of the C2 functional activity when preparing the C7 pool following DEAE-Sephacel chromatography (Table V). Factor B antigen was shown to co-chromatograph with C7 through the 2nd DEAE-Sephacel step but was effectively resolved by chromatography on CM-52 cellulose (Table V, Step B7; Fig. 6), eluting as a discrete component following C7 activity. The factor B in this pool retained biological activity as assessed by its ability to reconstitute factor B-depleted serum and lyse rabbit erythrocytes. C2 activity was shown to precede elution of C7 following the repeat DEAE-Sephacel step (Fig. 5). Following gel filtration on Sepharose 4B, the overall recovery of C7 was 19% of the starting plasma. The purification observed for C7 was 2270 and represents a gain in specific functional activity of almost 2-fold.

The purification of C8 from the post-DEAE-Sephacel pool (Table VI) also containing C6 and β1H was accomplished with only two additional steps: filtration on Sephadex G-200, followed by chromatography on CM-cellulose. The C8 preparation was purified only 547-fold as calculated from the specific activity increase shown in Table VI, Step B10. When analyzed by SDS-PAGE, the C8 preparation appeared pure as judged by Coomassie blue staining of the gel (Fig. 10d). Thus, we suspect that as much as 35% of the specific functional activity was lost. The overall recovery of C8 activity, however, was 32% of the activity present in plasma. Methods used here also allowed the recovery of both β1H and C6. The overall recovery of C6 activity was 38%. It is shown in Fig. 8 that β1H can be effectively resolved from C6 and C8 activities by gel filtration on Sephadex G-200. In preliminary experiments (not shown) the β1H recovered was further purified to homogeneity by isolation from an anti-β1H Sepharose immunoadsorbent. The product is biologically active as determined by its ability to participate in C3b inactivation.

The results of functional assays (Tables II and VII), immunologic tests, and SDS-PAGE analysis of the purified complement component preparations (Fig. 10), establish the high degree of purity obtained by the procedures described in this report. Thus, we have developed methodology which provides pure complement components in high yield from a single plasma pool. The availability of this protocol should facilitate further studies of the interaction and biological function of the various complement components.

Acknowledgments—We are grateful to Dr. Michael M. Frank for his critical comments and advice in preparation of the manuscript. We thank Dr. Marla Sautella for her assistance in preparation and titration of C5; Thelma A. Gaither, Kathy Katrou, and Mardell Wilson for her assistance in the typing of this manuscript.

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18, 1490–1497
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1131–1139
122, 277–298
767–776
140, 1324–1334
16, 2008–2015
111, 302
SUPPLEMENTAL MATERIAL

Large scale isolation of functionally active components of the human complement system

Erl H. Finne, George H. Wita, Loin Hofer, Mattie R. Sneed, and Brian F. Yack

TABLE III

Summary of C3 purification

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<th>Step</th>
<th>Volume</th>
<th>Conc.</th>
<th>Total</th>
<th>Yield</th>
<th>Conc.</th>
<th>Total</th>
<th>Yield</th>
<th>Specific</th>
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TABLE IV

Summary of C3 purification

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Fig. 2. Sepharose C3-4B gel filtration of the 2X PEG concentrated post-DEAE Sepharcl C3 pool (15 ml) on a 12 x 90 cm column bed. Equilibration and elution buffer: 150 mM NaCl, 1 mM EDTA, and 50 mM Tris-HCl, pH 7.4. Flow rate: 150 ml/hr; 2 ml/tube. The elution profile of C3 containing fractions was determined by C1r/C1s, HEG, and cl execution. C3 containing fractions were pooled as indicated by horizontal bar.

Fig. 3. Sepharose C3-4B gel filtration of low 2X PEG concentrated post-DEAE Sepharcl C3 pool (15 ml) on a 12 x 90 cm column bed. Equilibration and elution buffer: 50 mM NaCl, 50 mM EDTA, and 50 mM Tris-HCl, pH 7.4. Flow rate: 150 ml/hr; 2 ml/tube. The elution profile of C3 containing fractions was determined by C1r/C1s, HEG, and cl execution. C3 containing fractions were pooled as indicated by horizontal bar.

Fig. 4. Hydrophilic chromatography of the concentrated post-Sepharose C3-4B C3 pool on a 1.5 x 26 cm column bed. Equilibration and wash buffer: 50 mM NaCl, 50 mM EDTA, and 50 mM Tris-HCl, pH 7.4. Flow rate: 150 ml/hr; 2 ml/tube. The elution profile of C3 containing fractions was determined by C1r/C1s, HEG, and cl execution. C3 containing fractions were pooled as indicated by horizontal bar.

Fig. 5. Hydrophilic chromatography of the concentrated post-Sepharose C3-4B C3 pool on a 1.5 x 26 cm column bed. Equilibration and wash buffer: 50 mM NaCl, 50 mM EDTA, and 50 mM Tris-HCl, pH 7.4. Flow rate: 150 ml/hr; 2 ml/tube. The elution profile of C3 containing fractions was determined by C1r/C1s, HEG, and cl execution. C3 containing fractions were pooled as indicated by horizontal bar.
Isolation of Human Complement Components

### Table V

<table>
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<th>Activity Concentration</th>
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<td>A200/A240</td>
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<td>(10^-2)</td>
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<tr>
<td></td>
<td></td>
<td>(10^-2)</td>
<td>(10^-2)</td>
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</table>

* Chromatography performed on a column 12 x 90 cm.
* Recovery adjusted for quantity used.

---

**Fig. 3.** DEAE-Sepharose chromatography of the 20% PEG concentrated post-DEAE-Sepharose (Fig. 2) C7 and Factor B (Fb) pool on a 2.5 x 90 cm column bed. Equilibrium and elution buffers: 1 M KCl (pH 7.5) containing 10 mM NaCl, 1 mM EDTA, and 0.001% gelatin (5.0 ml/min), and a terminal rinse of the same buffer with 250 mM NaCl (25 ml/min). Flow rate: 20 ml/h, 5 ml/tube. Dashed horizontal bars indicate region of C7 and Fb activity and antigen, respectively.

**Fig. 4.** CM-cellulose chromatography of the concentrated post-DEAE-Sepharose (Fig. 3) C7 and Factor B (Fb) pool on a 2.5 x 90 cm column bed. Equilibrium and elution buffer: 3 M KCl (pH 7.5) containing 77 mM NaCl, 1 mM EDTA, and 0.001% gelatin (5.0 ml/min). Flow rate: 20 ml/h, 3 ml/tube.

**Fig. 5.** Sepharose CL-6B gel filtration of the concentrated post-CM-cellulose C7 pool on a 2.5 x 90 cm column bed. Equilibrium and elution buffer: 3 M KCl (pH 7.5) containing 77 mM NaCl, 1 mM EDTA, and 0.001% gelatin (5.0 ml/min). Flow rate: 20 ml/h, 8 ml/tube. Dashed horizontal bar indicates region of Fb antigen.
### TABLE VI

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume</th>
<th>Protein</th>
<th>Activity</th>
<th>Purity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mL</td>
<td>Conc.</td>
<td>Total</td>
<td>Yield</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Conc.</td>
<td>Total</td>
<td>Yield</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Value</td>
<td>Value</td>
<td>Value</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(g/mL)</td>
<td>(g/mL)</td>
<td>(g/mL)</td>
</tr>
<tr>
<td>1.</td>
<td>Plasma</td>
<td>1,975</td>
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<td>200</td>
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<td>4.90</td>
<td>6.60</td>
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<td>12.1</td>
<td>31.9</td>
<td>100</td>
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<td>2.12</td>
<td>10.0</td>
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<td>2.</td>
<td>DEAE-sepharose&lt;sup&gt;a&lt;/sup&gt; (1L PEG precipitate)</td>
<td>120</td>
<td>31.5</td>
<td>3.00</td>
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<td></td>
<td></td>
<td></td>
<td>11.9</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>99.5</td>
<td>11.9</td>
<td>50</td>
</tr>
<tr>
<td>3.</td>
<td>Sephacryl S-200 (1L post S-200)</td>
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<td>0.353</td>
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<td></td>
<td></td>
<td>4.27</td>
<td>66</td>
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<td>7.95</td>
<td>66</td>
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<td>9.</td>
<td>Om cellulose</td>
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<td>1.332</td>
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<td>35.1</td>
<td>3.35</td>
<td>10.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Chromatography performed as described in Materials and Methods, Part A.

<sup>b</sup> Recovery adjusted for quantity used.

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**Fig. 8.** Sephacryl S-200 gel filtration of the 2DEP PEG precipitate post-DEAE-sepharose. (a) C5, and (b) C6. (a) S-200 on a 1.2 x 15 cm column (same). Equilibration and wash buffer: 50 mM sodium phosphate (pH 6.8) containing 150 mM NaCl (15 ml/hr). Flow rate: 0.5 ml/hr, 20 ml/step.

**Fig. 9.** Om cellulose chromatography of the 2DEP concentrated post-sephacryl S-200 C5 and C6 gels on a 1.2 x 25 cm column (same). Equilibration and wash buffer: 50 mM sodium phosphate (pH 6.8) containing 45 mM NaCl, and 0.005% gelatin (2.5 ml/hr). Elution buffer: 30% a linear salt gradient to 375 mM NaCl (30 ml/hr). Flow rate: 0.5 ml/hr, 20 ml/step.