

# HUMAN C4-BINDING PROTEIN

## I. Isolation and Characterization\*

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Human C4 and mouse Ss proteins show extensive structural homologies. They are antigenically related (1) and are composed of three polypeptide chains of similar molecular weights, linked by disulfide bonds (2, 3). In addition, it is very likely that the major histocompatibility complex of these species contains structural genes for both proteins (3-5). However, some functional differences between the Ss protein and the C4 hemolytic activity of mouse serum have been reported (6).

We recently described a new protein in mouse serum which forms complexes with the Ss protein, and also with C4 of human or guinea pig origin (7, 8). Because of the remarkable specificity of its interaction with Ss (C4) we named this protein Ss- or C4-binding protein, and suggested it might be a new complement component. This question could not be appropriately studied in mouse serum, whose complement system is still poorly characterized. Therefore we approached the problem by searching for a C4-binding protein (C4-bp)<sup>1</sup> analogue in human serum.

In this paper we report the isolation and characterization of a human serum protein that differs from all known complement components, which has properties very similar to those of mouse C4-bp.

### Materials and Methods

*Materials.* Dimethyl suberimidate, Aldrich Chemical Co., Inc., Milwaukee, Wis.; agarose and human transferrin, Behring Diagnostics, American Hoechst Corp., Sommerville, N. J.; Biorex 70, *N,N'*-methylene-bis-acrylamide, acrylamide, *N,N,N',N'*-tetramethylethylenediamine, ammonium persulfate, sodium dodecyl sulfate (SDS), Bio-Rad Laboratories, Richmond, Calif.; diisopropylfluorophosphate (DFP), Calbiochem, San Diego, Calif.; sucrose, Fisher Scientific Co.,

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<sup>1</sup> *Abbreviations used in this paper:* BSA, bovine serum albumin; C4-bp, C4-binding protein; C4D, serum from a patient genetically deficient in C4; CIE, crossed immunoelectrophoresis; DFP, diisopropylfluorophosphate; DGVB, isotonic veronal buffer containing glucose and gelatin; OVA, ovalbumin; PBS, phosphate-buffered saline; PEG, polyethylene glycol; RIE, rocket immunoelectrophoresis; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; Tf, transferrin.

Pittsburgh, Pa.; bovine serum albumin (BSA), Miles Research Laboratories, Inc., Kankakee, Ill.; Staphylococcus A (IgG-sorb), New England Enzyme Center, Tufts University, Boston, Mass.; Nonidet P-40 (NP-40), Shell Chemicals Co., London, England; polyethyleneglycol mol wt 4,000,  $\epsilon$ -aminocaproic acid, ovalbumin,  $\beta$ -galactosidase (*Escherichia coli*), catalase (beef liver), myoglobin (horse heart), alcohol dehydrogenase (yeast), phosphorylase a (rabbit muscle), Sigma Chemical Co., St. Louis, Mo.; bovine thrombin, Parke, Davis and Co., Detroit, Mich.; DEAE Sephadex A-50, CNBr-activated Sepharose 4B, Pharmacia Fine Chemicals, Inc., Piscataway, N. J.; heparin (liquaemin sodium), Organon, Inc., W. Orange, N. J.; DEAE-cellulose (DE-52), Whatman Ltd., Springfield Mill, Maidstone, Kent, England; fuchsin sulfite, Harleco, Gibbstown, N. J. IgG, IgM, human myeloma proteins, and  $\alpha$ 2-macroglobulin were gifts of Dr. E. Franklin, New York University School of Medicine, New York.

*Diluents.* Isotonic veronal-buffered saline, pH 7.4, ionic strength 0.145 M, containing 0.1% gelatin,  $1.5 \times 10^{-4}$  M  $\text{CaCl}_2$  and  $5 \times 10^{-5}$  M  $\text{MgCl}$  ( $\text{GVB}^{++}$ ), dextrose veronal-buffered saline ionic strength 0.075 M ( $\text{DGVB}^{++}$ ) and  $2 \times 10^{-3}$  M disodium EDTA were prepared according to usual procedures (9). Barbitol-EDTA, buffer pH 8.6, containing 23 mM sodium barbitol, 3.7 mM barbituric acid, and 2 mM EDTA. Phosphate-buffered saline (PBS) was from Grand Island Biological Co., Grand Island, N. Y.

*Purified Complement Components and Reagents.* C1 and C4 were purified to homogeneity as described (10, 11). C4 had a specific activity 140 times higher than in the original plasma. In 5.6% SDS polyacrylamide gel electrophoresis (PAGE), under nonreducing conditions, C4 showed a single band with an apparent mol wt of 200,000 and after reduction, the characteristic  $\alpha$ ,  $\beta$ , and  $\lambda$  chains were observed. C4b was prepared by adding 10  $\mu\text{g}$  of C1 to 400  $\mu\text{g}$  of C4, and incubating the mixture for 18 h at 4°C. Cleavage of the  $\alpha$ -chain of C4 was ascertained by SDS-PAGE under reducing conditions and by loss of hemolytic activity. Pure C3 was a gift from Dr. B. Tack, National Institutes of Health, Bethesda, Md. Sera from patients with C4 deficiency and C3b inactivator deficiency were gifts from Doctors H. Ochs, University of Washington School of Medicine, Seattle, Wash., and C. Alper, Harvard University School of Medicine, Boston, Mass., respectively.

Sepharose-4B coupled to C4b was prepared as described in (12).

*Antisera.* Rabbit anti-human C4, C3, IgG, Clq, Cls, Cls inactivator, Behring Diagnostics, American Hoechst Corp.; goat anti-human C4, Meloy Laboratories Inc., Springfield, Va.; Rabbit anti-transferrin (IgG fraction), Dako Laboratories, Copenhagen, Denmark; goat anti-IgM, Cappel Laboratories, Inc., Cochranville, Pa.; rabbit anti- $\alpha$ -2 macroglobulin, Bio-Rad Laboratories, antiserum to C3b-inactivator, Kent Laboratories, North Vancouver; British Columbia; anti-C1r was a gift from Dr. R. R. Porter. Anti- $\beta$ 1H was a gift from Doctors C. Alper and D. Fearon, Harvard University School of Medicine, Boston, Mass. Anti-high molecular weight kininogen was a gift from Dr. J. V. Pierce, National Institutes of Health, Bethesda, Md. Anti-ovalbumin was a gift from Dr. Z. Ovary, New York University School of Medicine, New York. The IgG fraction of the goat antiserum to C4 was prepared by chromatography on DEAE-cellulose.

*Preparation of Antiserum to Human C4-bp.* Fresh human serum (1.2 ml) was incubated with 1.5 mg of ovalbumin (OVA) anti-OVA immune precipitates prepared at equivalence and suspended in 0.6 ml of  $\text{DGVB}^{++}$  buffer. The mixture was incubated for 10 min at 37°C, then 0.2 ml of 0.1 M  $\text{Na}_2\text{H EDTA}$ , pH 7.6 was added, followed by centrifugation at 1,000 g for 10 min. 0.4 ml of the supernate was mixed with 0.3 ml of an IgG fraction of a goat antiserum to C4. After incubation at 37°C for 30 min, the mixture was spun down; the supernate was further treated with a second aliquot of the goat antiserum to C4, and the cycle repeated until precipitation ceased to occur. The precipitated material was pooled, washed five times with  $\text{DGVB-EDTA}$ , dispersed with the aid of a syringe and needle, and resuspended in 1.5 ml of 0.15 M  $\text{NaCl}$ . This was emulsified in complete Freund's adjuvant and injected in the four footpads of two 5- to 6-mo-old female rabbits. Bleedings were started on day 21. By crossed immunoelectrophoresis, using whole human serum as antigen, both antisera contained only antibodies to C4 and to C4-bp. The antisera were made monospecific by absorption with Sepharose 4B-C4b.

*Analytical Procedures: Immunoelectrophoresis.* Crossed (CIE) and rocket (RIE) immunoelectrophoresis were run as described (13-15). For CIE, the electrophoresis in the first dimension was performed at 10 V/cm at 10°C. When necessary, human transferrin was mixed with the antigen studied to serve as an internal marker. RIE was run at 2 V/cm at 10°C for 16 h. The levels of antigen were evaluated by measurement of the peak heights after staining. At least three

dilutions of standards were included in each slide to estimate the relative concentration of the unknowns.

*Polyacrylamide Gel Electrophoresis.* Slab gel polyacrylamide electrophoresis was performed as described by Laemmli (16). The stacking and separating gel combinations used were either 3 and 5% or 3 and 7.5%. Disc gel electrophoresis was carried out as described previously (17) in 3.5% polyacrylamide gels.

To calibrate the gels for molecular weight estimations under reducing conditions, the following protein markers were used:  $\beta$ -galactosidase (130,000), phosphorylase A (94,000), BSA (68,000), yeast alcohol dehydrogenase (37,000), and catalase (60,000).

As standards for the determination of the molecular weight of C4-bp in its unreduced form, we prepared a mixture of multimeric IgG by a modification of a previously described cross-linking procedure (18, 19). In a typical protocol, 25  $\mu$ l of 1.2 mg/ml dimethyl suberimidate was mixed with 0.2 ml of 5–10 mg/ml of specifically purified (anti-dinitrophenol) rabbit IgG in 0.2 M Tris pH 8.5. To this mixture we immediately added 0.2 ml of 2% SDS in the same buffer. The samples were then frozen in ethanol-dry ice, the mixtures stored at  $-10^{\circ}\text{C}$  for 24 h and incubated at  $37^{\circ}\text{C}$  for 2 h before treatment with 2% SDS-6 M urea and electrophoresis. In most cases four to five dense bands were obtained, corresponding to the monomeric and di, tri, tetrameric forms of IgG. In a similar way we obtained oligomeric forms of BSA. Gels were stained for carbohydrate content by the periodic acid-Schiff technique (20).

*Sucrose Gradient Ultracentrifugation.* 0.2 ml of protein samples were loaded on top of 5-ml tubes containing linear 10–40% sucrose gradients in DGVB-EDTA. Ultracentrifugation was carried out at 38,000 rpm for 20 h at  $4^{\circ}\text{C}$  using a SW50.1 rotor. The positions of  $\alpha$ 2-macroglobulin, IgM, C3, C4-bp, and transferrin in serum were determined by RIE, while IgG was monitored by radial immunodiffusion. When purified C4-bp was subjected to ultracentrifugation, purified human C3,  $\alpha$ 2-macroglobulin, myeloma IgG, and radiolabeled BSA were added to the sample immediately before the run. These proteins served as standards for the determination of the sedimentation coefficient of C4-bp.

*Radiolabeling of C4-bp.* This was performed according to a modification of the Hunter and Greenwood method (21): 50  $\mu$ l of 0.8 M Tris-HCl, pH 7.5 was added to 2 mCi of  $^{125}\text{I}$ , aqueous solution, New England Nuclear, Boston, Mass., followed by 200  $\mu$ l of purified C4-bp at 0.35 OD/ml and 25  $\mu$ l of chloramine-T (0.5 mg/ml). The mixture was incubated for 5 min, and 25  $\mu$ l of sodium metabisulfite (0.5 mg/ml), followed by 0.2 ml PBS, were added to terminate the reaction. All reagents were kept in ice. The labeled protein was dialyzed for 48 h against PBS. After removal from the dialysis bag, 2 mg of BSA in 0.1 ml of PBS were added to the sample.

*Immunoprecipitation of  $^{125}\text{I}$ -C4-bp/Anti-C4-bp Complexes.* This was performed as described by Kessler (22) using a suspension of formaldehyde-treated Staphylococcus A (Cowan I strain).  $^{125}\text{I}$  C4-bp was treated with increasing volumes of rabbit antiserum to C4-bp or normal rabbit serum. The samples were incubated for 20 min at room temperature, then 50  $\mu$ l at 10% Staphylococcus A suspension were added and the mixture incubated for an additional 20 min at room temperature. The tubes were centrifuged at 2,000 *g* for 20 min, and the pellets washed several times by centrifugation. After counting the radioactivity associated with the pellets, 150  $\mu$ l of 2% SDS-6 M urea was added and the suspension was heated for 3 min in boiling water vapors. The bacteria were removed by centrifugation, the supernates were counted, and recoveries estimated. The eluates were loaded in SDS-PAGE slabs under nonreducing or reducing conditions. After electrophoresis the gels were dried and radioautographed for 2–24 h by using Kodak X-omat R film (Eastman Kodak Co., Rochester, N. Y.).

*Affinity Chromatography of  $^{125}\text{I}$ -C4-bp.* This was done by incubating  $^{125}\text{I}$ -C4-bp in 0.01 M Tris, 0.025 M NaCl, pH 8.6, with increasing vol of a 25% suspension of Sepharose 4B-C4b or Sepharose 4B-BSA for 30 min at  $37^{\circ}\text{C}$ . The beads were extensively washed in the same buffer. The material bound to the beads was eluted by treatment with 2% SDS-6 M urea for 3 min at  $100^{\circ}\text{C}$  and analyzed on SDS-PAGE slabs, followed by radioautography.

## Results

*Interaction between C4-Binding Protein and C4 in Human Serum.* The formation of complexes between an activated form of C4 and C4-binding protein was first suggested when antisera monospecific to C4-bp and to C4 were used to

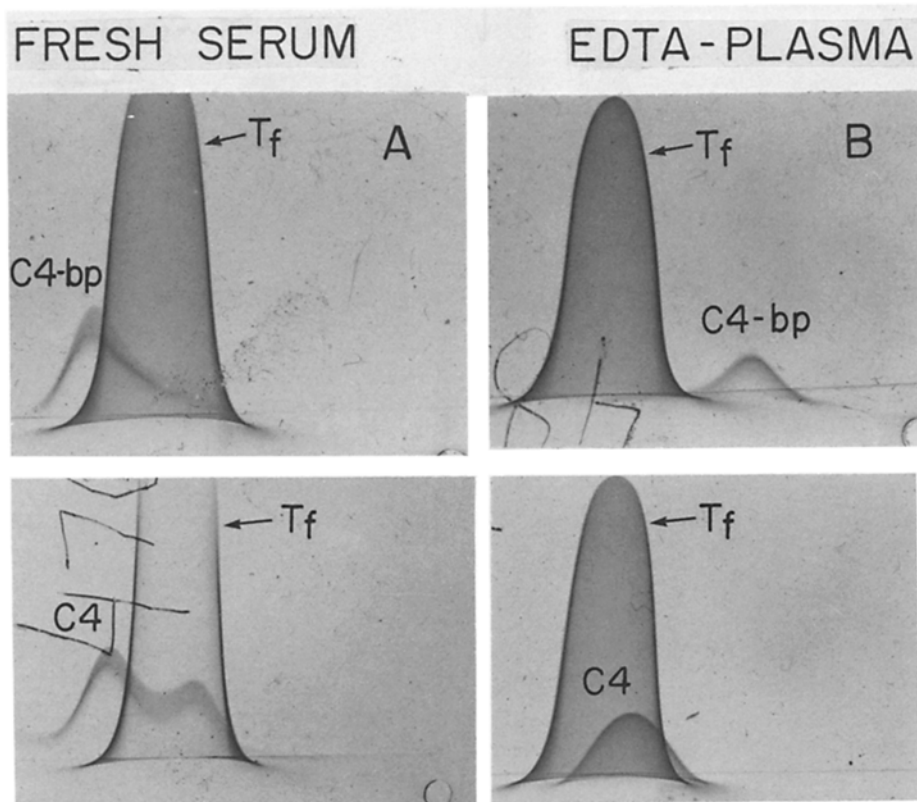


FIG. 1. CIE in 1% agarose, EDTA-barbital buffer, of serum and EDTA-plasma. After subjecting the plasma or serum to electrophoresis in the first dimension, the second dimension was performed in the presence of either antiserum to C4-bp (top slides) or to C4 (bottom slides). An antiserum to transferrin was added to all slides. The transferrin peak serves as an internal marker (Tf).

develop CIE of samples of human serum and EDTA-plasma.

After CIE of EDTA-plasma (Fig. 1, top right) C4-bp moves as a slow  $\beta$ -globulin. However, in serum (top left) under the same conditions, the position of the C4-bp peak shifts markedly toward the anode and is asymmetric, with some trailing toward the cathode (transferrin serves as an internal marker).

C4 patterns in the same samples are shown below. While in EDTA-plasma (Fig. 1, bottom right) C4 appears as a homogeneous peak which has the same mobility as purified C4, in serum C4 shows a bimodal distribution. The slower part of the C4 peak has an electrophoretic mobility similar to that of native C4 while the fast portion of the C4-peak coincides with the position of C4-bp. Although the ratios between slow versus fast C4 varied in different runs of various sera, in all experiments the C4-bp peak was shifted in relation to its position in EDTA-plasma, and its position always coincided with the position of the fast C4 peak.

The bimodal distribution of C4, as well as the anodal displacement of C4-bp, reflects the activation of the complement system during electrophoresis in agarose and the binding of activated C4 to C4-bp. The activation of C1 and

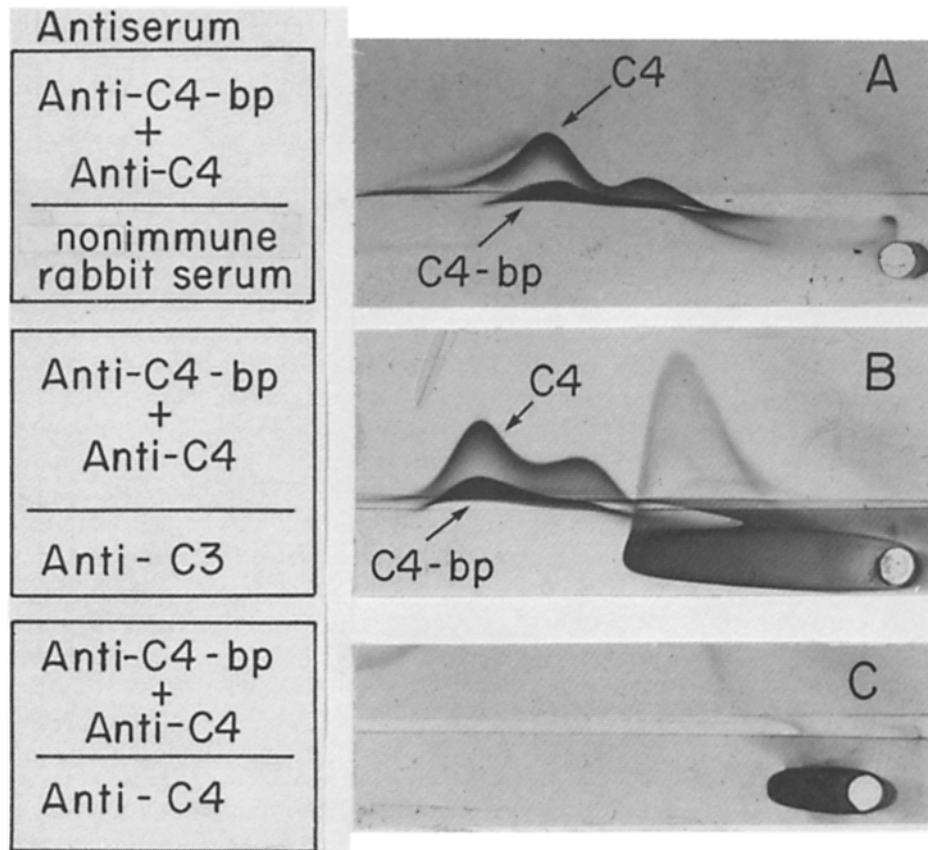


FIG. 2. Formation of activated C4/C4-bp complexes in serum. Samples of undiluted fresh human serum were subjected to CIE. The first dimension of electrophoresis was run in the presence of antisera to C4 (plate C), C3 (plate B), or normal rabbit serum (plate A). The second dimension was always performed in the presence of an oligo-specific rabbit antiserum containing antibodies to C4-bp and C4. In plate A, C4 and C4-bp migrate as fast  $\beta$ -globulins and their peaks overlap. In plate C, the presence of an antiserum monospecific to C4 during the first dimension completely abrogated both C4 and C4-bp peaks. In contrast, in plate B, the C4-bp and C4 peaks are not modified although a heavy C3 anti-C3 precipitate formed during electrophoresis in the first dimension.

proteolysis of C4 after contact of serum with agarose have previously been reported (23), and can be prevented by chelation of divalent cations. If 10 mM EDTA were added to serum before CIE, C4 and C4-bp showed profiles identical to those in EDTA-plasma. That activated C4 and C4-bp form complexes in serum was demonstrated by a technique involving a combination of RIE and CIE (Fig. 2). In the first part of the experiment, human serum was subjected to RIE in the presence of a monospecific rabbit antiserum to C4 (plate C) or of normal rabbit serum (plate A). The plates were then subjected to the second electrophoresis (CIE) in agarose containing a mixture of antibodies to C4-bp and C4. In plate C, the presence of anti-C4 in the first dimension completely abrogated both C4 and C4-bp peaks. To rule out the possibility that C4-bp had bound nonspecifically to the C4-anti-C4 precipitate formed in the first dimen-

sion, a control plate was included in which an antiserum to C3 (instead of anti-C4) was added to the agarose. Although a strong C3-anti-C3 precipitate appeared during RIE, the peaks of C4 or C4-bp were not affected (plate B), when compared to those shown in plate A.

To analyze the interaction between activated C4 and C4-bp, we proceeded to isolate C4-bp.

*Isolation of C4-Binding Protein.* C4-bp was isolated from plasma by a combination of chromatographic procedures on DEAE-Sephadex, Biorex-70, and precipitation by polyethyleneglycol. The fractionation of C4-bp was monitored by RIE using monospecific antisera to C4-bp and to C4. All steps of the fractionation were carried out at 4°C.

60 ml of 0.1 M EDTA, pH 7.55, was added to 540 ml of fresh acid citrate dextrose plasma. The plasma was stirred in ice and precipitated by drop-wise addition of 300 ml of 15% (wt/vol) of polyethyleneglycol dissolved in phosphate-buffered saline, 15 mM EDTA, pH 7.55. After 20 min, the plasma was centrifuged at 10,000 *g*. The precipitate was resuspended to 600 ml with 0.01 M Tris, 0.18 M NaCl, pH 7.6. The insoluble material was removed by centrifugation, and to the supernate we added calcium chloride to 2 mM, 400 U of thrombin, and the mixture was incubated at 37°C for 10 min. The resulting clot was liquified in a Waring blender for 5 s and centrifuged at 10,000 *g* for 15 min. To the supernate we added drop-wise half volume of a 26% solution of polyethyleneglycol made in the same buffer as above. After 20 min at 0°C, the precipitated fraction was dissolved in 100 ml of a buffer containing 0.01 M Tris, pH 8.6, 0.075 M NaCl, 5 mM EACA, and 5 mM EDTA, centrifuged to remove insoluble material and loaded on a 2.7 x 35-cm column of DEAE Sephadex A-50 equilibrated with the same buffer. The column was washed with the starting buffer until the OD 280 reached 0.080, and then eluted with 900 ml of a linear NaCl gradient (Fig. 3).

C4-bp antigen eluted in three major peaks between 8 and 16 mS (at 0°C). The first peak, which did not contain appreciable amounts of C4, was pooled and concentrated by precipitation with one half volume of 26% polyethyleneglycol (PEG), as described above. The precipitate was resuspended in 25 ml of 0.02 M phosphate buffer, pH 7.2, with a conductivity of 1.8 mS at 0°C, cleared of insoluble material by centrifugation, and applied to a Biorex-70 column (2.5 x 30 cm) equilibrated with the same buffer. The column was washed with the starting buffer and then a linear NaCl gradient was applied. C4-bp eluted at conductivities above 10 mS, as a heterogeneous peak (Fig. 4, peaks A, B, and C). Peak A contained C4, in addition to C4-bp. Peaks B and C were pooled and concentrated to a vol of 13 ml ( $E_{280\text{nm}}^{1\text{cm}} = 0.35$ ). When analyzed on SDS-PAGE, the pooled fractions showed two major bands, which migrated very close to each other. These bands were in an identical position to those seen in the individual fractions from the column, although the fractions contained different proportions of the two protein bands (Fig. 4). In addition, two minor contaminants of lower molecular weight could be seen in the individual fractions as well as in the pool.

That the two major bands in the pooled fractions were C4-bp was shown by two independent criteria: they bind specifically to C4b, and are recognized by the monospecific antiserum to C4-bp, as shown by the following experiment. Purified C4-bp was radiolabeled and aliquots subjected to two different treat-

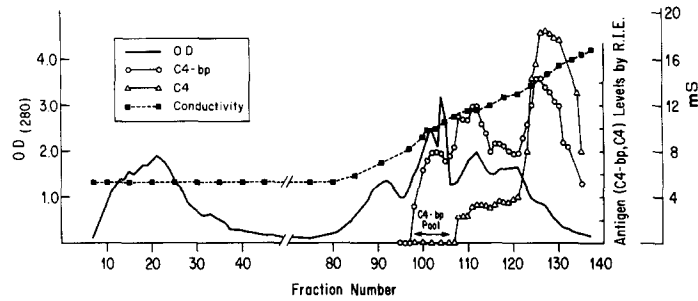


FIG. 3. Chromatography on DEAE-Sephadex. The 0-5% polyethyleneglycol fraction of plasma was recalcified, clotted with thrombin, and the clot removed as described in the text. The supernate was precipitated with PEG. The precipitate was resuspended in 0.01 M Tris, 0.075 M NaCl, 5 mM EACA, 5 mM EDTA, pH 8.6, conductivity 5.2 mS (0°C) and loaded on a DEAE-Sephadex A-50 column (2.7 x 35 cm) equilibrated with the same buffer. After elution of the pass-through peak, the column was washed and a linear NaCl gradient was applied. The mixing chamber contained 450 ml of the starting buffer, and it was connected by a syphon to a flask containing 450 ml of eluting buffer, the conductivity of which was adjusted to 20 mS with NaCl. 6-ml fractions were collected at a rate of 20 ml/h. C4 and C4-bp were monitored by RIE.

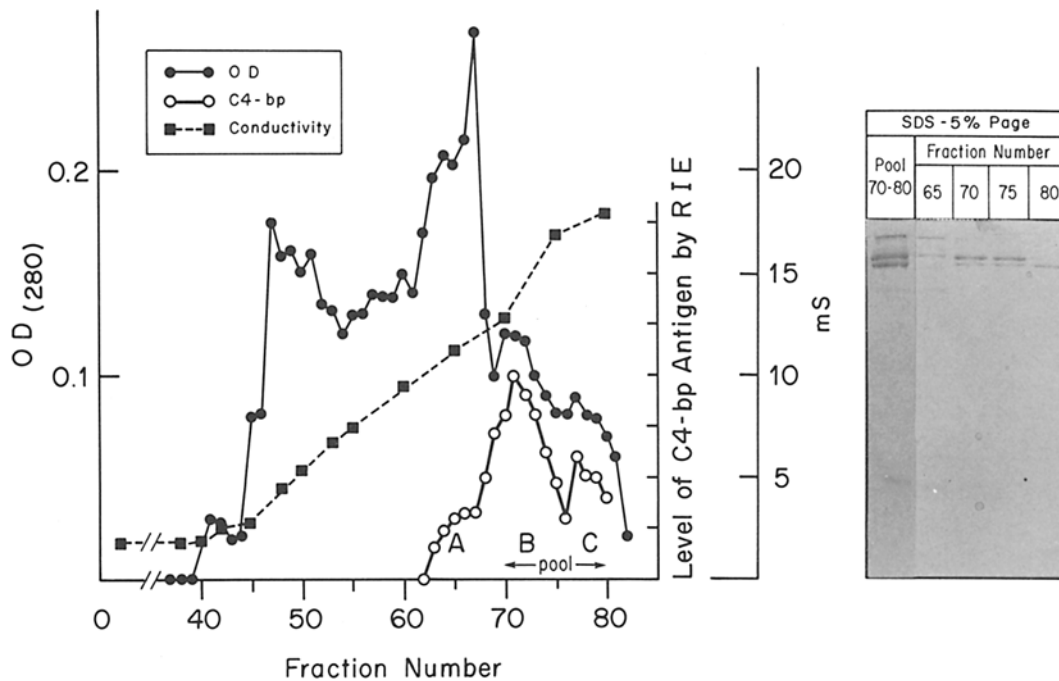


FIG. 4. Biorex-70 chromatography. The C4-bp pool from DEAE-Sephadex chromatography was concentrated by addition of half volume of 26% PEG and resuspended in 25 ml of 0.02 M phosphate buffer pH 7.2 at a conductivity of 1.8 mS (0°C). The sample was applied to a Biorex column (2.5 x 30 cm) equilibrated with the same buffer. After washing, C4-bp was eluted with a linear salt gradient. The mixing chamber contained 400 ml of the starting buffer and the second flask contained 400 ml of 0.7 M NaCl in the same buffer. 4-ml fractions were collected at a rate of 20 ml/h. C4-bp eluted as a heterogeneous peak (A, B, C). Fractions 65, 70, 75, 80, and the pooled 70-80 fractions were analyzed by 0.1% SDS, 3%/5% slab PAGE.

ments: (a) incubation with Sepharose-4B beads coupled either to pure human C4b or bovine serum albumin as a control. The beads were washed several times with 0.01 M Tris, 0.025 M NaCl, pH 8.6, then eluted with a solution containing 2% SDS and 6 M urea. The eluates were subjected to SDS-PAGE under nonreducing and reducing conditions with 2-mercaptoethanol; (b) incubation with antiserum to C4-bp or with normal rabbit serum. The immune complexes were removed from solution with formaldehyde-treated *Staphylococcus A*. The bacteria were washed extensively and treated with 2% SDS-6 M urea to elute and dissociate the antigen antibody complexes. Again, the eluates were counted and subjected to SDS-PAGE under nonreducing and reducing conditions.

In both experiments between 55 and 60% of the counts were specifically bound to the particles. That the same material was involved in both cases was shown by cross absorption experiments; less than 5% of the counts were immune precipitated by the antiserum to C4-bp if the sample had been pretreated with an excess of Sepharose 4B-C4b.

The proteins which bound to the beads were further identified after radioautography of the stained, dried SDS-PAGE gels. Before reduction (left side of Fig. 5), the specific eluates obtained from both experiments contained only the two heavy bands shown in the starting sample. After reduction (right side of Fig. 5), a single specific band of mol wt 70,000 daltons was observed. The mol wt of the subunit of C4-bp was also determined in experiments in which mixtures of purified, radiolabeled C4-bp and purified C4 were reduced and subjected to 5.6% SDS-PAGE. After staining the gels and radioautography, we found that the C4-bp band coincided with that of the  $\beta$ -chain of C4, which has a mol wt of approximately 70,000 daltons.

*Properties of Purified C4-Binding Protein.* C4-bp appears to be distinct from other known complement components and serum proteins. It is present in the serum of patients with genetic deficiencies in C2, C4, C3b-inactivator, or high molecular weight kininogen. C4-bp did not react with monospecific antisera to the following serum proteins: Clq, Clr, Cls, C4, C3, C1-inhibitor,  $\beta$ 1H, C3b-inactivator,  $\alpha$ -2 macroglobulin, and high molecular weight kininogen.

The nature of the structural differences between the two bands of C4-bp is not known. Both bands stain heavily with the periodic acid-Schiff reagent, suggesting high contents of carbohydrate. As mentioned, after complete reduction in 2-mercaptoethanol and 6 M urea, a single polypeptide chain of 70,000 daltons is seen in SDS-PAGE (Fig. 5). The mol wt of the two major bands before reduction were calculated to be 590,000 and 540,000 daltons, using as standards oligomers of rabbit IgG and human IgM (Fig. 6), or BSA oligomers (not shown). From these data we infer that C4-bp consists of several disulfide bonded protomers (perhaps eight) of 70,000 daltons.

By sucrose gradient ultracentrifugation, purified C4-bp sedimented at 10.7 s. To compare in a more rigorous fashion the sedimentation coefficients of purified C4-bp and serum C4-bp, the purified protein was radiolabeled and 2  $\mu$ g were added to 0.2 ml of serum from a patient genetically deficient in C4. The mixture was subjected to ultracentrifugation and the fractions monitored for radioactivity and for C4-bp antigen by RIE. As shown in Fig. 7 A the peak of radiolabeled



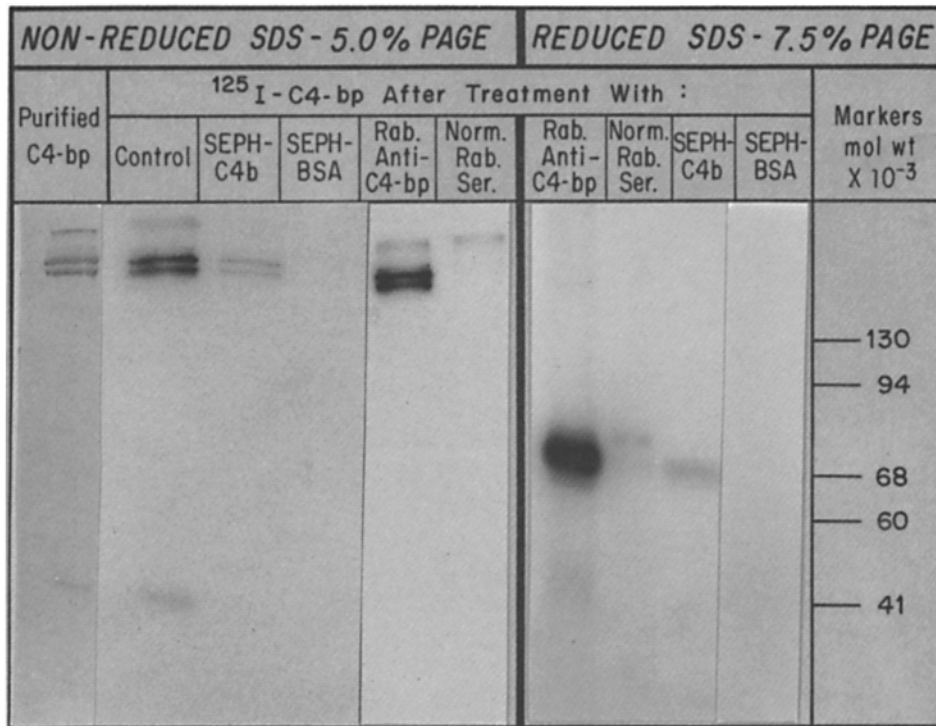


FIG. 5. Identification by immunological and functional criteria of C4-bp after electrophoresis on SDS-PAGE. Fractions 70-80 from Biorex chromatography were pooled, concentrated, and radiolabeled. The specific activity was  $2 \times 10^6$  cpm/ $\mu$ g protein. C4-bp was identified both by immunoprecipitation and affinity chromatography. For immunoprecipitation,  $3 \times 10^6$  cpm of <sup>125</sup>I-C4-bp in Tris buffer 0.01 M, pH 8.6 containing 0.025 M NaCl and 1 mg/ml BSA were mixed with increasing volumes of antiserum to C4-bp (1, 2, 4, 8, 16, and 32  $\mu$ l) or with normal rabbit serum. 50  $\mu$ l of a 10% suspension of Staphylococcus A were added to each reaction mixture and incubated for 20 min at room temperature. After centrifugation at 2,000 g for 20 min, the pellets were washed several times. After counting the washed pellets, 150  $\mu$ l of 2% SDS-6 M urea were added and the bacteria suspension was heated for 3 min in boiling water vapors. After spinning down at 2,000 g for 20 min, the supernate was removed, counted, and recoveries estimated. A maximum of 55% of the labeled material was specifically immunoprecipitated. The eluates were subsequently loaded in SDS-PAGE slabs under nonreducing conditions, or after reduction with 20%  $\beta$ -2 mercaptoethanol. After electrophoresis the gels were stained, dried, and radioautographed. The figure (left to right) illustrates that both major bands on the partially purified <sup>125</sup>I C4-bp are specifically recognized by anti-C4-bp. After reduction (right part of the figure) and electrophoresis, a single polypeptide chain of 70,000 daltons is obtained. The C4-bp band was functionally identified by incubating  $6 \times 10^5$  cpm of <sup>125</sup>I-C4-bp with increasing amounts of pelleted Sepharose 4B-C4b or Sepharose 4B-BSA. After several washes with 0.01 M Tris, 0.025 M NaCl pH 8.6, the labeled material was eluted from the beads with 2% SDS-6 M urea. 60% of the label was specifically bound to the beads. Analysis of the eluates on SDS-PAGE gels under nonreducing or reducing conditions is illustrated. Again the two major bands of the C4-bp preparation bound specifically to Sepharose 4B-C4b and after reduction, a single polypeptide chain of 70,000 was obtained. The markers on the right side of the figure were included in the 7.5% gels only.

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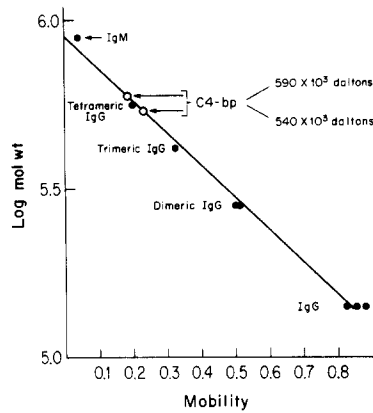


FIG. 6. Estimation of the molecular weight of C4-bp by SDS-PAGE. Molecular weights were measured by disc gel electrophoresis according to the method of Weber and Osborn (17). Markers were: human IgM and oligomeric forms of rabbit IgG. Gels were 0.1% SDS and 3.5% polyacrylamide.  $r^2 = 0.99$ ,  $P < 0.01$ .

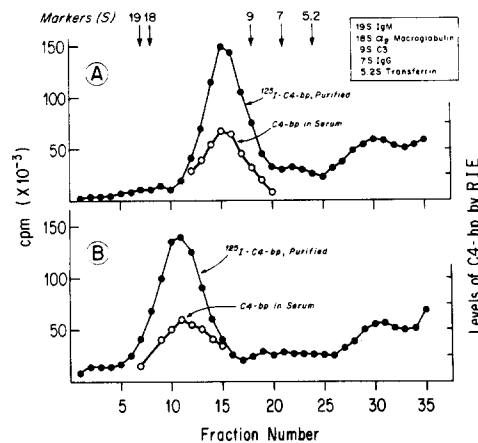


FIG. 7. Comparison of purified C4-bp and C4-bp in C4-deficient (C4D) serum by sucrose gradient ultracentrifugation.  $^{125}\text{I}$ -C4-bp ( $10 \mu\text{l}$ , containing about  $2 \mu\text{g}$  protein and  $3 \times 10^6$  cpm) were mixed with 0.4 ml of C4D serum. 0.15 ml of this mixture was incubated at  $37^\circ\text{C}$  for 10 min with either (A) 0.15 ml of 0.01 M Tris, 0.025 M NaCl, pH 8.6, or (B) 0.15 ml of purified C4b (0.7 mg/ml) in the same buffer. 0.2 ml of each mixture was loaded on top of 5 ml 10–40% sucrose gradients and ultracentrifuged at 38,000 rpm for 20 h. The sedimentation coefficient of C4-bp was calculated as 10.7 S using known proteins as standards. ( $r^2 = 0.996$ ,  $P < 0.01$ ). The standard proteins were added to C4-bp before the run and their positions in the gradient after centrifugation were determined by RIE.

purified C4-bp coincided precisely with that of C4-bp from serum. Furthermore, the addition of purified C4b to the C4D-serum (Fig. 7 B) resulted in a parallel displacement of both peaks to the bottom of the gradient (15.3 s).

In Fig. 8 we demonstrate that C4-bp, purified (top, right) or in C4D-serum (bottom, right) moves in electrophoresis as a slow  $\beta$ -globulin in barbital-EDTA buffer. In buffer containing  $\text{Ca}^{++}$ -lactate, C4-bp moves as a gamma globulin (not shown). A marked shift in its mobility is caused by addition of purified C4b

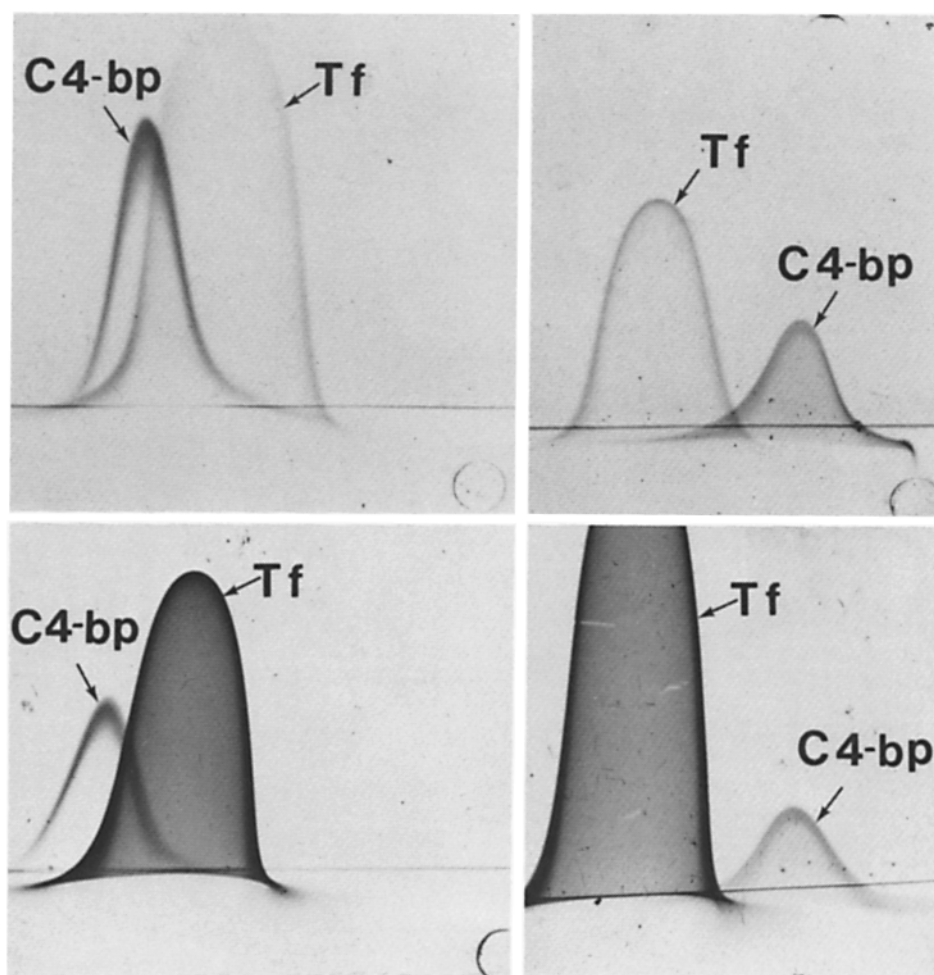


FIG. 8. Anodic shift of purified C4-bp after interaction with purified C4b. Purified C4-bp and C4-bp in C4D serum migrate as slow  $\beta$ -globulins (right, top and bottom). Upon incubation with purified C4b, serum C4-bp and purified C4-bp shifted toward the anode (left, top, and bottom). Top right, C4-bp purified 120  $\mu\text{g}/\text{ml}$ ; bottom right, C4D serum diluted 1:4; top left, C4-bp purified, 120  $\mu\text{g}/\text{ml}$  + C4b, 200  $\mu\text{g}/\text{ml}$ ; bottom left, C4D serum diluted 1:4 + C4b, 200  $\mu\text{g}/\text{ml}$ . The diluent was barbital-EDTA. The mixtures were incubated at 37°C for 1 h and 5  $\mu\text{l}$  of each sample was subjected to CIE. Transferrin was added to the mixtures containing purified C4 and C4-bp. The slides were developed with a mixture of antisera to C4-bp and to transferrin (internal marker).

(top and bottom, left). The new position of C4-bp coincides with that of C4-bp in normal human serum (compare Figs. 1 and 8). Furthermore, the formation of complexes between purified C4-bp and C4b, was shown by a combination of RIE and CIE, as described in Fig. 2; antisera to C4 completely precipitated C4-bp in the mixtures (not shown).

*Stoichiometry of the C4b/C4-bp Interaction.* The formation of C4/C4-bp complexes using purified proteins can be easily detected by CIE. As shown in Fig. 8, C4-bp, which is a slow  $\beta$ -globulin, moves much faster toward the anode

## HUMAN C4-BINDING PROTEIN

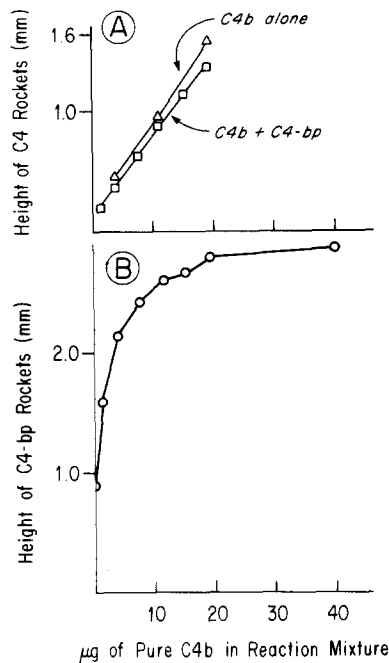


FIG. 9. Purified C4-bp rocket heights vary according to the concentration of C4b added to the samples. Aliquots of purified C4-bp (50  $\mu$ l, containing 12.2  $\mu$ g of purified protein) were mixed with 100  $\mu$ l of barbital-EDTA containing increasing concentrations of purified C4b. The mixtures were incubated for 1 h at 37°C and analyzed for either C4-bp or C4 antigen by RIE. The bottom graph (B) illustrates that rocket heights of C4-bp are strongly influenced by the concentration of C4b in a given sample. Graph A (top) shows that the C4b rockets are not affected by the interaction with C4-bp. An absorption coefficient value of  $E_{280\text{nm}}^{1\text{cm}} = 1$  for C4-bp at a concentration of 1 mg/ml was assumed.

after interaction with C4b. Furthermore, complex formation leads to a marked increase in the heights of C4-bp rockets obtained after CIE or RIE. For example, in Fig. 8 the area of bound C4-bp is much greater than the area of C4-bp alone.

The differences in the properties between free and bound C4-bp can be used to estimate the stoichiometry of its reaction with C4b. In the experiment shown in Fig. 9 B, increasing amounts of C4b were added to a constant amount of C4-bp. The mixtures were incubated at 37°C for 60 min and then subjected to RIE. As illustrated, the heights of the rockets increased gradually and reached a plateau. The amount of C4b necessary for maximum rocket heights was found to be equal to the amount needed for a complete shift in the position of the C4-bp peak after CIE. This amount of C4b probably saturates all sites of C4-bp. If we assume the mol wt of C4b and C4-bp to be 190,000 and 560,000 daltons, respectively, and the extinction coefficient for C4-bp at 1 mg/ml to be 1.0, saturation is reached at a ratio 4.8 molecules of C4b per molecule of C4-bp.

The increase in the height of the C4-bp rockets upon complex formation with C4b reflects a modification of the antigenic determinants of C4-bp as recognized by the antiserum used in this experiment. Part of the determinants of C4-bp may have been covered by C4b, and in addition new determinants exposed,

since this antiserum was obtained by immunization with C4b/C4-bp complexes (see Materials and Methods).

When C4 antigen was measured by RIE, the results obtained varied according to the antisera used. With some antisera, the heights of rockets were greatly changed after complex formation with C4-bp. In other cases (Fig. 9 A) very little or no change was observed.

These findings highlight one possible pitfall in the measurement by immunological methods of the concentration of proteins which form complexes with other molecules. Because of these difficulties, we could not calculate the recoveries and specific activities of C4-bp during the purification procedure, nor could we determine its levels in human serum.

### Discussion

In this paper we describe the isolation and characterization of a protein in human serum which displays specific binding affinity for the activated form of C4 (C4b). This protein, named C4-bp, differs from all known complement components.

C4-bp was purified from plasma by PEG precipitation, followed by sequential ion-exchange chromatography. The recovery and specific activity of C4-bp after fractionation could not be calculated, for the reasons described in the Results section.

Purified C4-binding protein is a 10.7 s glycoprotein. It migrates as a slow  $\beta$ -globulin after agarose electrophoresis in barbital-EDTA buffer. However, when electrophoresis is performed in the presence of Ca-lactate, purified C4-bp migrates as a gamma globulin. Its apparent mol wt on SDS-PAGE is 540–590,000 daltons. It consists of several identical subunits of 70,000 daltons linked by disulfide bonds.

Analysis of the purified preparation by SDS-PAGE revealed the presence of two major bands and two minor contaminants. The two major bands were identified as C4-bp by functional and immunological criteria: both forms of C4-bp bound specifically to Sepharose 4B-C4b, and were immunoprecipitated by monospecific antisera. The small difference in molecular weight of the two forms of C4-bp is probably not artefactual, and reflects a true heterogeneity. For example, although both bands were detected in peaks B and C obtained after chromatography on Biorex-70 (Fig. 4), the high mol wt C4-bp predominated in peak B, while the lower mol wt form predominated in C. This observation suggests that these two forms of C4-bp have slightly different net electric charges at pH 7.2. The reason for this heterogeneity is unknown. Gross denaturation as a consequence of the purification procedure is unlikely since the purified material retained all properties of C4-bp in serum. The two forms of C4-bp may represent structural variants, since these are detected even after a single-step (euglobulin) serum fractionation. The precise nature of this variation remains to be elucidated.

Purified C4-bp binds to purified C4b. Complex formation did not require additional co-factors and was directly demonstrated by ultracentrifugation in sucrose gradients (Fig. 7) and by CIE. During electrophoresis in agarose at pH

8.6, the complexes moved faster toward the anode than C4-bp alone (Fig. 8) and could be coprecipitated by an antiserum specific for C4.

Complexes formed in the presence of diisopropylfluorophosphate ( $10^{-5}$  M) or EDTA ( $10^{-2}$  M). Similarly, as previously reported, the binding of mouse C4-bp to human C4b could not be inhibited by DFP, soy bean trypsin inhibitor, ovomucoid, or by chelation of divalent cations (8).

Based on the progressive shift in the electrophoretic mobility of C4-bp, as well as on the parallel changes in its antigenic determinants after interaction with C4b, we could evaluate the stoichiometry of the reaction. We found that C4-bp molecules are multivalent and each can bind a maximum of four to five molecules of C4b. However, precise determination of the valence of C4-bp and of the stoichiometric relationships, must await accurate measurement of the molecular weight of C4-bp.

The data presented in this paper may be helpful in clarifying the conflicting results presented in the literature on the electrophoretic mobility patterns of C4 in serum and EDTA-plasma. In agreement with Sjöholm and Laurell (23), we found that during electrophoretic runs of serum in agarose containing media, the complement system is activated, probably due to C1 conversion to C1 $\bar{I}$  (24). This activation leads to fragmentation of C4 to C4b, and complex formation with C4-bp. As shown, these complexes display altered electrophoretic mobility as compared to free C4b and C4-bp. Therefore, at least three bands containing C4 antigen may be formed; that is, native C4, C4b, and C4b/C4-bp complexes. If the complexes contain different ratios of C4b to C4-bp, the situation may be even more complex. In contrast, in EDTA-plasma, C1 activation is prevented and stable complexes are not formed.

Recently, Teisberg et al. (5) have reported data on the genetic polymorphism of C4, using samples of blood collected in heparin. We found that the formation of C4/C4-bp complexes after electrophoresis in agarose is prevented by the addition of 25 U or more of heparin/ml of blood. When smaller doses are used, stable complexes may still form.

The role of C4-bp in the complement cascade and in modulating C4 activity will be the subject of a separate communication.

### Summary

C4-binding protein (C4-bp), a new component of the complement system, was isolated from human plasma by precipitation with polyethyleneglycol, followed by chromatography on ion exchangers. C4-bp was identified on sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) by two independent criteria: its ability to bind to C4b, and immunoprecipitation with a monospecific antiserum.

Purified C4-bp is a 10.7 s glycoprotein. It consists of several disulfide bonded subunits of mol wt 70,000 daltons. Under nonreducing conditions, its mol wt has been estimated on SDS-PAGE as 540-590,000 daltons.

C4-bp moves as a slow  $\beta$ -globulin at pH 8.6 in the absence of free divalent cations, but when the buffers contain Ca $^{++}$ -lactate, C4-bp is a gamma globulin.

Purified C4-bp binds to purified C4b. The reaction proceeds in the presence or absence of divalent cations and is not inhibited by diisopropylfluorophosphate.

The C4b/C4-bp complexes have sedimentation coefficients between 15 and 17 s on sucrose gradient ultracentrifugation, and can be readily identified by crossed immunoelectrophoresis (CIE). The complexes move faster toward the anode than either protein.

C4-bp is multivalent. Saturation is reached at molecular ratios of C4b/C4-bp of between 4 and 5.

The interaction between C4b and C4-bp may complicate the electrophoretic patterns of these proteins in normal human serum, if the complement system is activated before or during the run. However, in EDTA-plasma, native C4 and C4-bp do not form stable complexes and can be identified in separate peaks after CIE.

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