MULTIPLE SEDIMENTING SPECIES OF PROPERDIN IN HUMAN SERUM AND INTERACTION OF PURIFIED PROPERDIN WITH THE THIRD COMPONENT OF COMPLEMENT*‡

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Properdin was originally described by Pillemer and co-workers in 1954 as a unique serum protein participating in an alternative pathway of complement (C) activation (1). Over the next several years, the sedimentation behavior of partially purified preparations of properdin was examined in several laboratories and values of 27S, 18S, 10S, and 5–7S were variously reported (1–5). Finally, in 1968 Pensky and co-workers obtained a homogenous preparation of properdin which sedimented at 5.2S in the analytical ultracentrifuge (6). Although earlier discrepancies could be attributed at least in part to methodological limitations, the possibility could not be excluded that the differences might also have been on a more substantive basis, perhaps related to the degree of purification achieved. With the development in our laboratory in 1973 of a radioimmunoassay for the detection of nanogram quantities of properdin (7), sucrose density gradient ultracentrifugation experiments were performed to determine the sedimentation behavior of properdin antigen in normal human serum. Radiolabeled or unlabeled properdin purified to homogeneity according to the method of Pensky et al. (6) served as control. The results, presented in this paper, provide evidence for the existence of multiple sedimenting species of properdin antigen in serum and identify the third component of C (C3) as a constituent of at least one of the heavier sedimenting species.

Materials and Methods

Human Serum. Whole blood was obtained from healthy laboratory volunteers or was purchased from a commercial distributor (Knickerbocker Biologicals, Inc., New York), clotted, and centrifuged in the cold to separate the serum. Portions were used fresh or stored frozen at −70°C. Serum from a patient with partial lipodystrophy was supplied by Dr. Robert McLean, University

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of Connecticut Health Center, Farmington, Conn. Serum from a patient genetically deficient in C3 was supplied by Doctors Chester Alper and Fred Rosen, Children's Hospital Medical Center, Boston, Mass.

Human Plasma. Blood from healthy donors (7 ml) was drawn into glass vacutainers containing 70 μl of 15% K_3EDTA solution (3204 QS; Becton, Dickinson & Co., Orangeburg, N. Y.). Centrifugation at 3,000 rpm and 10,000 rpm sequentially separated cells and platelets, respectively, from plasma.

Buffers. Phosphate-buffered saline (PBS),[1] pH 7.4, ionic strength 0.15 μ, was used as is or fortified with 5×10^{-3} M EDTA by diluting with a stock solution of 0.15 M EDTA, pH 7.4. PBS, ionic strength 0.075 μ was made by diluting PBS (vol/vol) with deionized water; PBS, ionic strength 0.45 μ was made from 10 times stock solution of PBS. Where indicated, divalent metal cations Ca^{2+} and Mg^{2+} were present at 1.5×10^{-4} M and 5×10^{-4} M, respectively.

Antisera. Rabbit antisera to human C3, properdin, and factor B were prepared with purified antigens after a general immunization regimen described elsewhere (7). When necessary, antisera were rendered monospecific by immunoabsorption with human gamma globulin (American Cyanamid Co., Lederle Laboratories, Pearl River, N. Y.). Native or absorbed antisera were stored at −20°C. Goat antiserum to whole human serum was purchased (Meloy Laboratories, Inc., Springfield, Va.).

Purified Proteins. Human activated properdin ($\bar{P}$) was purified from pooled normal serum by the zymosan adsorption and column chromatographic method described by Pensky et al. (6).

Human C3 was prepared by modification (8) of the method of Nilsson and Müller-Eberhard (9). Human C3b was generated by incubating purified C3 with serum-exposed washed cobra venom factor (CVF) sepharose beads (10). Low molecular weight by-products of the reaction were separated from C3b by chromatographing on a calibrated Sephadex G-200 column. C3b eluted in a homogeneous peak at a position corresponding to a mol wt of 162,000 daltons.

Human C3c was prepared according to the protocol for the purification of C3 with the following changes. To generate C3c in serum, the alternative pathway of C was activated by pretreating the starting serum with zymosan (4 mg/ml, lot no. 7B-340, Fleishchmann Laboratories, Standard Brands, Inc., New York) at 37°C for 20 min. During the zymosan activation step no precautions were taken to inhibit C3b inactivator. The first column chromatographic step (DEAE) was conducted using buffer 10 times more concentrated in EDTA than indicated in the protocol (8,9). C3c eluted on the descending side of the dropthrough peak and was recycled on the same column before proceeding to the second column step. The mol wt of purified C3c was 110,000 daltons (10) as determined by analytical sodium dodecyl sulfate disc gel electrophoresis (kindly done by Ronald J. Field, Children's Hospital Medical Center, Boston, Mass.). Human Factor B (C3 proactivator) was purified as described previously (11).

CVF was isolated from crude cobra venom (lyophilized, *Naja naja*, Venom Laboratory, Silver Springs, Fla.) by DEAE-cellulose chromatography (microgranular, DE52, Whatman; H Reeve Angel & Co., Clifton, N. J.). CVF eluted in a linear salt gradient; the starting buffer was phosphate buffer, pH 7.3, 1.9mS (23°C) fortified with chloramphenicol 5×10^{-5} M and the limit buffer was 0.5 M NaCl in starting buffer. Fractions containing CVF were identified by screening for anticomplementary activity using a modification of the method of Ballow and Cochrane (12).

Concentration of Protein Solutions. This was accomplished on Amicon PM10 ultrafiltration membranes (Diaflo; Amicon Corp., Scientific Sys. Div., Lexington, Mass.) in pressurized (40–70 lb/in^2 nitrogen), stirred cells at cold room temperatures.

Solid-Phase CVF. Covalent linkage of CVF to sepharose 4B (CNBr activated; Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) was accomplished by mixing 10 mg CVF with 2 g swollen beads in 0.1 M borate buffer, pH 8.6, containing 0.5 M NaCl. The slurry was incubated for 2 h at room temperature and overnight at 4°C. Free CNBr sites were blocked with 1 M ethanolamine, pH 8.0. Nonspecifically absorbed protein was removed by sequential washes with 0.1 M acetate buffer, pH 4, containing 1 M NaCl and 0.1 M borate buffer, pH 8.6, containing 1 M NaCl. Sepharose-CVF was suspended in PBS for use.

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[1] *Abbreviations used in this paper:* BGG, bovine gamma globulin; BSA, bovine serum albumin; CVF, cobra venom factor; CTY C, cytochrome C; $\bar{P}$, human activated properdin; PBS, phosphate-buffered saline.

Molecular Weight Markers. Substances used to calibrate Sephadex G-200 columns were Blue Dextran (2000, Pharmacia Fine Chemicals, Inc., Piscataway, N. J.), bovine gamma globulin (BGG) (160,000 daltons, Fraction II, bovine plasma; Armour Laboratories, Armour and Company, Chicago, Ill.), bovine serum albumin (BSA) (69,000 daltons, Fraction V powder, Sigma Chemical Company, St. Louis, Mo.), and cytochrome C (CYT C) (12,500 daltons, horse heart, Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.). Sedimentation positions in sucrose density gradients were marked with thyroglobulin (19.2S) (porcine, Type II, Sigma Chemical Company), BGG (7.3S), BSA (4.5S), ovalbumin (3.5S) (2× Cryst, Schwarz/Mann Div., Becton, Dickinson & Co.), and CYT C (1.5S). Radiolabeled or unlabeled human properdin sedimented at approximately 6S under the conditions employed for sucrose density gradient ultracentrifugation.

Solid-Phase Radioimmunoassay for Human Properdin. This was performed by major modifications of a published method (7). Two variations of the solid-phase radioimmunoassay were used, the sequential technique and the equilibrium technique. In view of the central importance of these assays in the experiments to be described, the precedures and data analyses are described in some detail.

Polystyrene tubes (12 × 75 mm, Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) were coated at room temperature for 2 h with 1 ml of diluted monospecific rabbit antiserum to human properdin (1:1,000 in 0.06 M sodium barbital buffer, pH 9.6). Unbound antibody was aspirated. According to schedules that will be delineated, the tubes were filled with solutions (1 ml) containing unlabeled properdin (unknown sample), radiolabeled iodine-125 properdin (^{125}I-$\bar{P}$), or a mixture of both. PBS buffer fortified with 1% BSA and 0.02% sodium azide served as diluent. For the equilibrium radioimmunoassay, solutions of unlabeled properdin (sample) and a standard concentration of ^{125}I-$\bar{P}$ (10–20 ng $\bar{P}$/ml, ~800–1800 cpm/ml) were mixed, transferred to antibody-coated tubes, and incubated; for the sequential radioimmunoassay the tubes were filled first with sample and incubated, then aspirated, and then refilled with the standard concentration of ^{125}I-$\bar{P}$ and reincubated. Various dilutions of a control serum pool, assumed to contain a normal serum concentration of properdin (25 μg $\bar{P}$/ml), were included in selected assays to serve as a reference curve with which to compare samples. Equilibrium conditions between antibody irreversibly bound to the tube wall and antigen were established after incubation overnight (16–24 h) in a temperature-controlled (37°C ± 1°C), humidified incubator (Double chamber incubator, Model 17060-00Z, National Appliance Co., Portland, Ore.). Additional tubes that held only the standard concentration of ^{125}I-$\bar{P}$ were placed directly into plastic carrier tubes and counted 10 min for gamma emission; the average total count (T) was expressed in counts per minute. For all remaining tubes, including several that contained only ^{125}I-$\bar{P}$, the free antigen was separated from the bound antigen by aspirating and discarding the liquid contents (free antigen) before counting the tubes. For each tube that had contained sample antigen the corresponding counts per minute were designated bound counts (B). The average counts per minute of the tubes that had contained only ^{125}I-$\bar{P}$ at the standard concentration was referred to as maximum bound counts (B_0). In all of the assays reported in this paper, the ratio $B_0/T \times 100$ was between 68–100%.

In some experiments the amount of properdin in samples was expressed simply as the percent ratio B/T symbolized $B/T-\%$ ($B/T \times 100$). A plot of the logarithm of the microliters of the properdin-containing serum or of the nanograms of P against the arithmetic values of $B/T-\%$ gave a sigmoidal function. Over a considerable range of this curve, the values plotted as a straight line with slope m. The ratio of potencies between two properdin-containing solutions, one giving a value of $B_1/T-\%$ and the other $B_2/T-\%$, was then equal to the antilog $\{[(B_1/T-\%) - (B_2/T-\%)]/m\}$. In a limited number of experiments all $B/T-\%$ values were related to $B_0/T-\%$, the maximum uptake value, by the formula, antilog $\{[(B_0/T-\%) - (B/T-\%)]/m\}$, and the data were recorded as relative concentration of properdin. In assays that included both experimental samples and dilutions of the control serum, it was possible to report the data as nanograms of properdin. In these cases, a logit transformation was employed. The B values corresponding to the dilutions of the control sera were first converted to Y values by the formula $Y = B/B_0$ and then Y values were converted to logit values $Y/1 - Y$. The points (nanograms P, $Y/1 - Y$) when plotted on 2-cycle log-log scale yielded a set of points through which a straight line could be interpolated. The equation of the straight line permitted the calculation of nanograms of properdin (ng P) for each $Y/1 - Y$ value representing experimental samples. To facilitate the calculations a programmable calculator (Model 500, Wang Laboratories, Inc., Tewksbury, Mass.) was used.

For each freshly labeled batch of ^{125}I-$\bar{P}$, the standard concentration of properdin was determined from a standard curve. 1-ml vol of increasing concentrations of ^{125}I-$\bar{P}$ were incubated under assay conditions in antibody-coated tubes. Tubes were counted for gamma emission for 10 min (T), aspirated, and the emptied tubes were recounted (B). The resultant standard curve of B vs. T plotted on log-log scale progressed from a linear curve at lower concentrations of ^{125}I-$\bar{P}$ to a hyperbolic curve at higher concentrations of ^{125}I-$\bar{P}$. The T value of the point of transition from the linear to the hyperbolic region on the standard curve was taken to be the standard concentration of ^{125}I-$\bar{P}$.

Radioiodination of Properdin. Purified properdin was labeled with ^{125}I according to the iodine monochloride method of Helmkamp et al. (13). The radiolabeled protein was buffered in PBS containing 0.5% BSA, sterilely filtered (0.45 μm disposable filter unit, Millipore Corp., Bedford, Mass.) and stored at refrigerator temperatures (+4–8°C).

Measurement of Radioactivity. Polystyrene assay tubes or glass tubes (12 × 75 mm) were placed into 16 × 125 mm plastic carrier tubes (Amersham/Searle Corp., Arlington Heights, Ill.) and counted for gamma radiation at optimal instrument settings in a scintillation well counter (Model 4230, Nuclear-Chicago Corporation, Des Plaines, Ill.).

Sucrose Density Gradient Ultracentrifugation. Density gradient ultracentrifugation was performed by a modification of the method of Martin and Ames (14), described by Pensky et al. (6), employing an L2-65B Model Ultracentrifuge and SW 41 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). Gradient fractions containing marker proteins were diluted with 0.3 ml of PBS and measured for protein concentration at OD 280 nm in a 1 cm path in a Zeiss PMQII Spectrophotometer (Carl Zeiss, Inc., New York). Portions of gradient fractions containing serum or purified C proteins were analyzed for protein by the Folin method (15).

Hemolytic assay for C3. The relative concentration of C3 in fractionated serum was assayed using the intermediate complex EAC4, prepared by modification of the method of Borsos and Rapp (16), and KBr-treated serum as a reagent lacking C3 and C4 (17,18). This modification was developed by Mr. Ira Goodkofsky in our laboratory. Veronal-buffered saline, pH 7.4, ionic strength 0.15 μ, containing 1.5×10^{-4} M Ca^{2+}, 5×10^{-4} M Mg^{2+}, and 0.05% gelatin served as diluent and was prepared as described elsewhere (19). Reaction mixtures containing 0.1 ml of EAC4 (3.3×10^8/ml), 0.2 ml of KBr-treated serum diluted 1/25, and 0.2 ml of serial dilutions of sample were incubated at 37°C for 30 min. After the addition of 2 ml of chilled buffer, the tubes were centrifuged and the supernatant fluids read at 415 nm to measure hemolysis. The reciprocal of the dilution of sample that resulted in 50% hemolysis was taken as the relative concentration of C3.

Results

Observation of Multiple Sedimenting Species of Properdin in Human Serum and Plasma. As shown in Fig. 1, properdin antigen in normal serum subjected to sucrose density gradient ultracentrifugal analysis separated into one peak and two shoulders with approximate sedimentation coefficients of 6S, 9S, and 12S. For comparison, purified ^{125}I-$\bar{P}$ sedimented homogeneously at about 6S, a finding in accord with previously published reports (6,20). The total serum proteins were resolved as expected into 4S, 7S, and 19S peaks. The distribution of properdin in the 6S, 9S, and 12S species in serum under these conditions was 8, 36, and 56%, respectively, as estimated by integration of the area under each peak after the logarithmic ordinate of Fig. 1 was converted to a linear scale.

To exclude the possibility that multiple sedimenting species of properdin constituted an isolated finding unique to a single serum, sera from several different donors were analyzed under similar experimental conditions. Essentially indistinguishable results were obtained. Furthermore, no difference occurred when the gradients were made in PBS without added Mg^{2+} or when the analyzed samples were fresh serum, serum that had been stored frozed at −70°C, or serum that had been dialyzed against PBS.

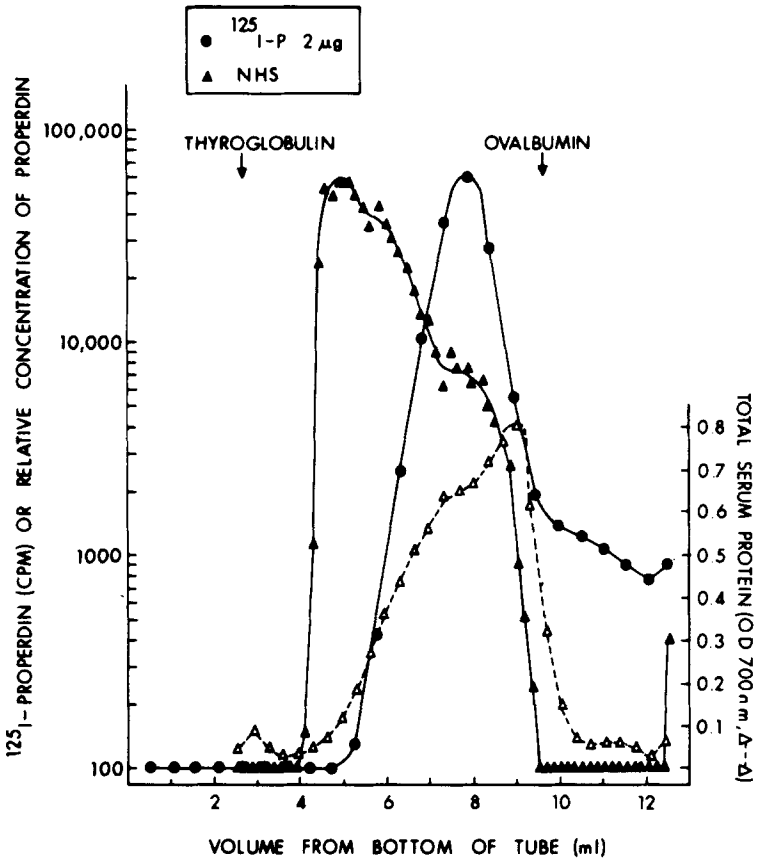

FIG. 1. Properdin antigen in normal human serum (NHS) compared with purified ^{125}I-P̄. NHS (0.5 ml) and ^{125}I-P̄ (4 μg/ml) were layered on 10–40% linear sucrose density gradients made in PBS containing 5×10^{-4} M Mg^{2+}, and were separated by ultracentrifugation 40,000 rpm, 2°C, 29 h). Thyroglobulin (19.2S) and ovalbumin (3.5S) served as markers.

The phenomonon was not an effect secondary to blood coagulation nor was it influenced by chelation of divalent cations since the properdin antigen profiles were similar in EDTA plasma and serum. Concentrating serum twofold by Amicon ultrafiltration and layering increasing volumes of either concentrated or native serum onto gradients failed to alter the distribution of properdin antigen originally observed when 0.5 ml of native serum was applied to the gradient.

In these and in subsequent gradient experiments to be described, no properdin antigen in serum was ever detected at positions lighter than 6S. This serves as presumptive evidence that ~6S properdin antigen in serum and ~6S highly purified properdin are similar molecular entities. Heavier sedimenting species of properdin antigen in serum, amounting to approximately 90% of the total properdin in serum, might, therefore, represent polymers of properdin, protein complexes between properdin and other serum proteins, or conceivably, a combination of polymerization and protein complex formation. Accordingly, the effect

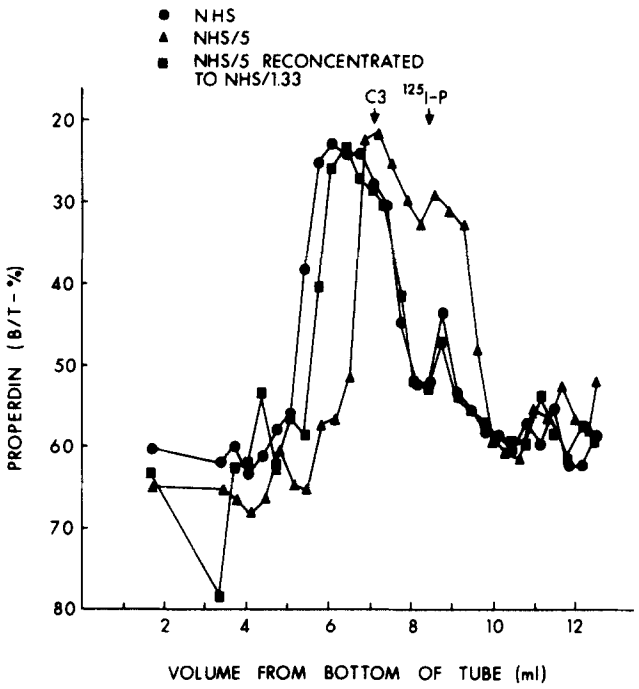

FIG. 2. Effect of dilution of serum on the sedimentation of properdin antigen. Equal vol (0.5 ml) of serum samples were layered onto 10–40% sucrose gradients made in PBS. Ultracentrifugation at 37,000 rpm was conducted at 2°C for 28 h. The positions (↓) of C3 and ^{125}I-$\bar{P}$ were obtained from another gradient experiment run under identical conditions.

of several parameters known to influence protein-protein interactions was investigated.

Effect of Dilution, Ionic Strength, and Temperature on the Multiple Sedimenting Species of Properdin in Serum. Dilution of serum by as little as 1/2.5 with PBS buffer fortified with 0.5% BSA before sucrose gradient fractionation occassioned loss of 12S properdin and a concomitant increase in concentration of both 9S and 6S properdin. As shown in Fig. 2, the effect was reversible. Serum that had been diluted 1/5 with PBS was subsequently reconcentrated to 1/1.33 by Amicon ultrafiltration. The relative concentration of 6S, 9S, and 12S properdin in reconcentrated serum assumed proportions similar to those seen in the original serum.

Dialysis of serum against pH 7.4 PBS of ionic strengths 0.075, 0.15, and 0.45 μ was without effect on the distribution of total serum proteins in sucrose gradients (right-hand panel of Fig. 3). However, profound effects were apparent on the various sedimenting species of properdin antigen (left-hand panel of Fig. 3); lower ionic strengths induced heavier sedimenting species of properdin antigen while higher ionic strengths favored lighter sedimenting species of properdin antigen. In this experiment fewer gradient fractions were subjected to radioimmunoassay, resulting in poorer resolution of properdin species in Fig. 3 as compared with Fig. 1. Nevertheless, at physiologic ionic strength of 0.15 μ, properdin antigen appeared to be distributed as ~6S, 9S, and 12S species. A

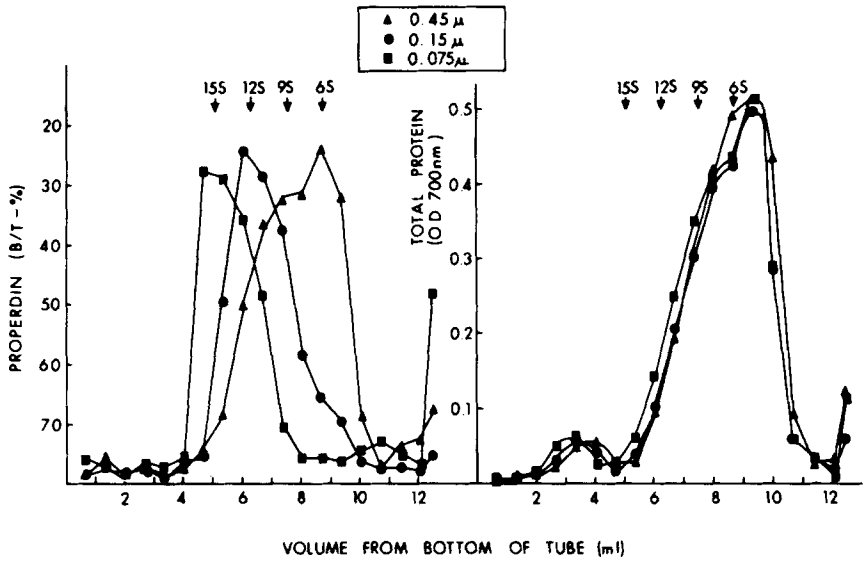

FIG. 3. Effect of serum ionic strength on the sedimentation of properdin antigen. Serum (0.5 ml) in pH 7.4 PBS of ionic strengths 0.075, 0.15, and 0.45 μ were centrifuged (37,000 rpm, 2°C, 29 h) on 10–40% gradients made in the respective buffers. Indicated sedimentation positions (↓) were interpolated from a standard sedimentation coefficient line obtained from a previous experimental run conducted under identical conditions.

threefold increase in ionic strength resulted in reduction in concentration of 12S properdin and an increase in both 6S and 9S properdin. Conversely, reducing the ionic strength to one-half physiologic ionic strength effected a disappearance of 6S and 9S properdin, reduction of 12S properdin and the appearance of a heavier species of properdin antigen sedimenting at ~15S.

In order to investigate the effect of temperature on the distribution of properdin antigen in human serum, 0.5 ml of serum was layered on a 10–40% linear sucrose gradient in PBS (pH 7.4; μ, 0.15) and centrifuged at 41,000 rpm for 26 h at 23°C. Under these conditions properdin was found only in the 6S and 9S positions, suggesting that dissociation of the 12S peak had occurred.

As shown by the effects of varying serum concentration, ionic strength, and temperature, the sedimentation behavior of properdin antigen in serum displayed characteristics of protein-protein dissociation-association reactions. The evidence supported the concept that properdin in serum exists as multimolecular complexes. The nature of the protein or proteins with which properdin interacts was therefore next investigated.

Sedimentation of Properdin Antigen in Serum from Patients with Genetic Deficiency of C3 and with Partial Lipodystrophy. Among the hypotheses that could explain the observations in the preceding sections was the possibility that properdin forms multimolecular complexes with other constituents of the alternative pathway. It was therefore of interest to examine the distribution of properdin antigen in sera from patients with two kinds of C3 abnormality. Indeed, as compared with normal serum, there was marked difference in the sedimentation of properdin in serum genetically lacking C3 (21) (Fig. 4). This

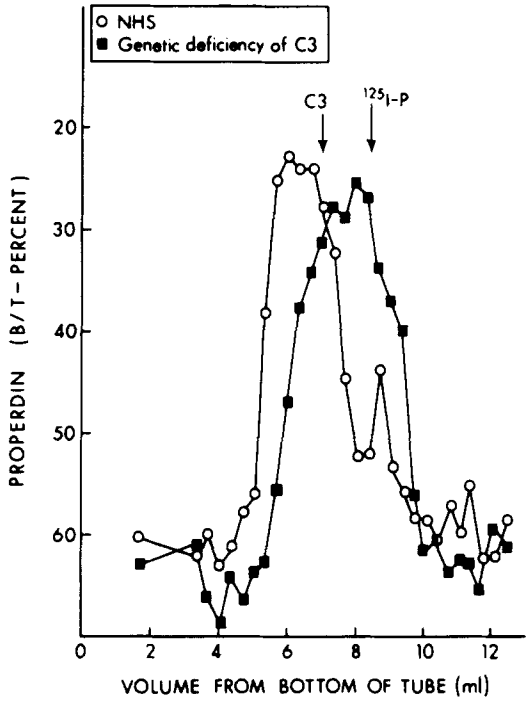

FIG. 4. The sedimentation of properdin antigen in genetically C3-deficient serum. Genetically C3-deficient serum (0.5 ml) and normal human serum (NHS) (0.5 ml) were centrifuged (37,000 rpm, 2°C, 28 h) on 10–40% gradients made in PBS. The positions (↓) of C3 and ^{125}I-P were obtained from another gradient experiment run under identical conditions.

serum is totally deficient in C3 but contains other known constituents of the alternative pathway. Properdin sedimented in a broad peak at approximately 7S, a position intermediate between normally present 6S and 9S properdin. Furthermore, when partial lipodystrophy serum, which contained C3 nephritic factor and lacked detectable C3 antigen (less than 3% normal C3 concentration), was fractionated on sucrose gradients, properdin antigen again sedimented at predominantly ~7S (left-hand panel of Fig. 5). The patient's serum showed a relatively normal total protein distribution (right-hand panel of Fig. 5). For comparison, normal human serum again contained ~6S, 9S, and 12S properdin. It therefore appeared that C3 might be a protein with which properdin interacts and the experimental approach was shifted to examination of the sedimentation properties of purified properdin in the presence or absence of purified C3.

Sedimentation of Purified Properdin Alone or in the Presence of Various Concentrations of Purified C3, C3b, or C3c. Sucrose gradient fractionation was utilized to study the role of C3 and two catabolic products of C3, C3b and C3c, on the sedimentation of purified properdin. Chilled solutions of the appropriate purified proteins were mixed at cold room temperatures (4–8°C) and the resultant mixtures were applied directly to cooled gradients just before centrifugation. As shown in the left-hand panels of Figs. 6–8, in the three mixtures containing the highest molar ratios of C3, C3b, or C3c to properdin (29.1/1), that

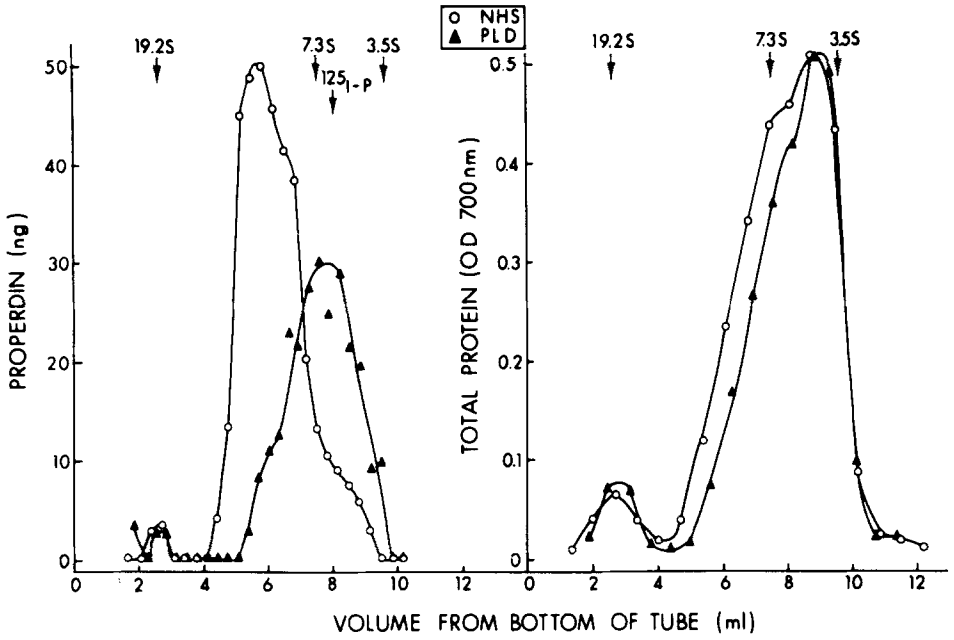

FIG. 5. The sedimentation of properdin antigen in partial lipodystrophy serum. Partial lipodystrophy serum (PLD) (0.5 ml) and normal human serum (NHS) (0.5 ml) were centrifuged (41,000 rpm, 2°C, 26 h) on 10–40% gradients made in PBS. Thyroglobulin, BGG, ovalbumin, and ^{125}I-P̄ served as markers (↓).

is, at ratios lower than but approaching concentrations existing in native serum, purified properdin shifted from its ~6S position to heavier gradient positions. At the highest ratios of C3 or C3b to properdin (left-hand panels of Figs. 6 and 7, respectively), properdin localized in the same position occupied by the respective homogeneous protein peaks, corresponding to values of ~9S and ~8.7S (right-hand panels of Figs. 6 and 7, respectively). However, at the highest ratio of C3c to properdin, properdin sedimented at ~8.2S, a position in the gradient that corresponded to the region of asymmetry on the heavier side of the respective protein curve (right-hand panel of Fig. 8). At lower molar ratios of C3, C3b, or C3c to properdin (11.6/1, 5.8/1, and 2.9/1) distinct peaks of properdin sedimented with coefficients intermediate between expected values. The expected heavier value was previously defined by the position of properdin in mixture with the highest molar ratio of C3, C3b, or C3c to properdin (29.1/1), and the expected light value at approximately 6S, the control position of purified properdin alone. The possibility that the intermediate sedimentation values could have been ascribed to artifactual error was ruled out. Total protein analysis of the gradients which monitored only the C3 species of protein in the mixtures (the smaller quantities of properdin were below the level of detection of the assay) showed that in all mixtures (right-hand panel of Figs. 6–8) the positions of the protein peaks varied only within the limits of experimental error. Therefore, technical aberrations introduced into the gradients could not serve as the basis for a simple explanation of the intermediate sedimenting species of properdin. A theoretical basis for the observed complex sedimentation pattern of properdin in

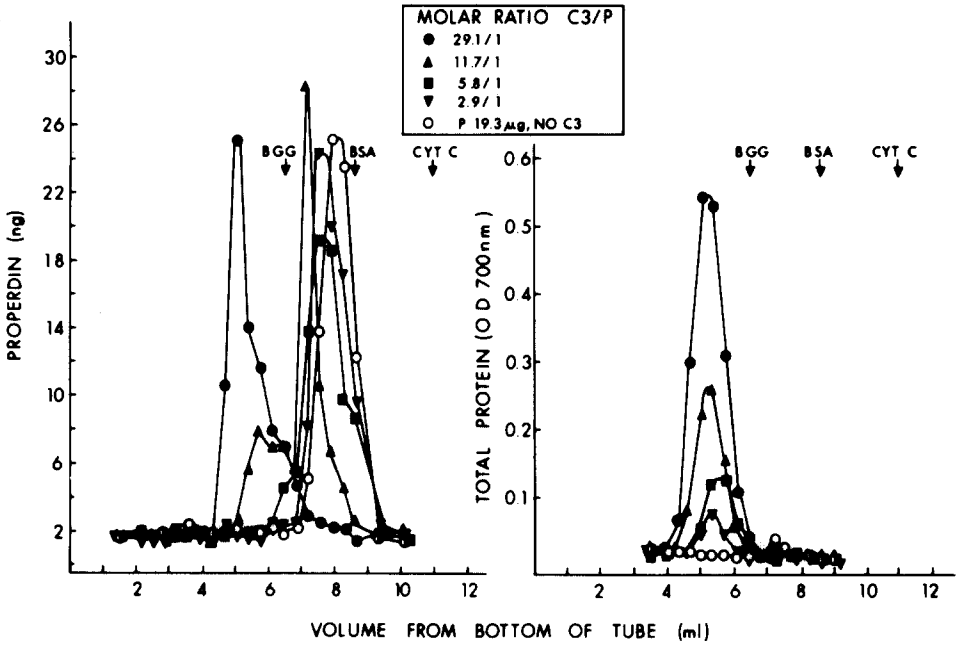

FIG. 6. The sedimentation of purified properdin in the presence or absence of various concentrations of purified C3. Equal vol (0.5 ml) of properdin (control) or mixtures of properdin and C3 were centrifuged (41,000 rpm, 2°C, 17 h) on 1–15% gradients made in PBS. BGG, BSA and CYT C served as markers (↓).

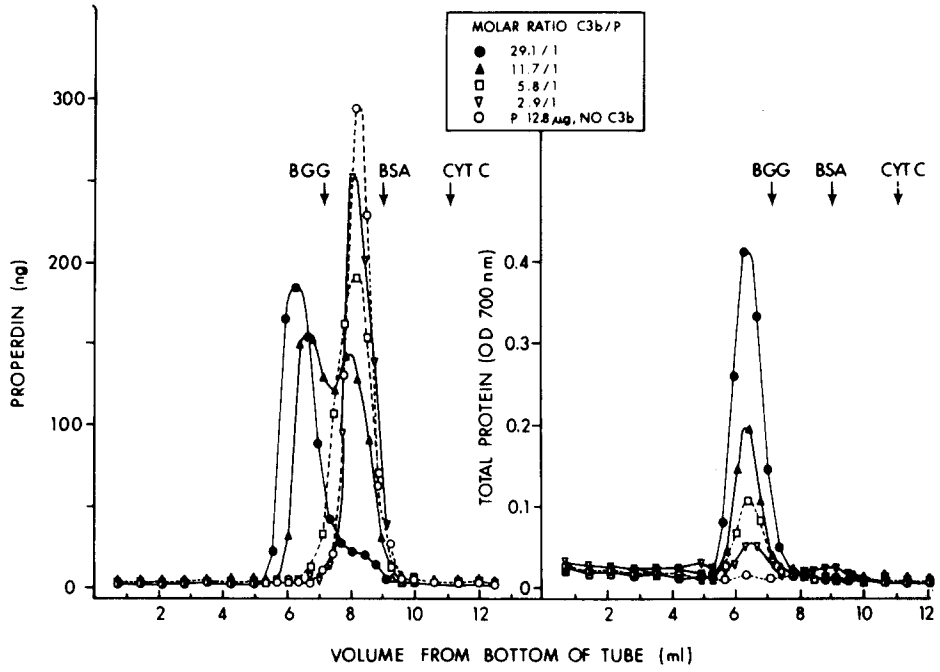

FIG. 7. The sedimentation of purified properdin in the presence or absence of various concentrations of purified C3b. Equal vol (0.5 ml) of properdin (control) or mixtures of properdin and C3b were centrifuged (41,000 rpm, 2°C, 15 h) on 1–15% gradients made in PBS. BGG, BSA, and CYT C served as markers (↓).

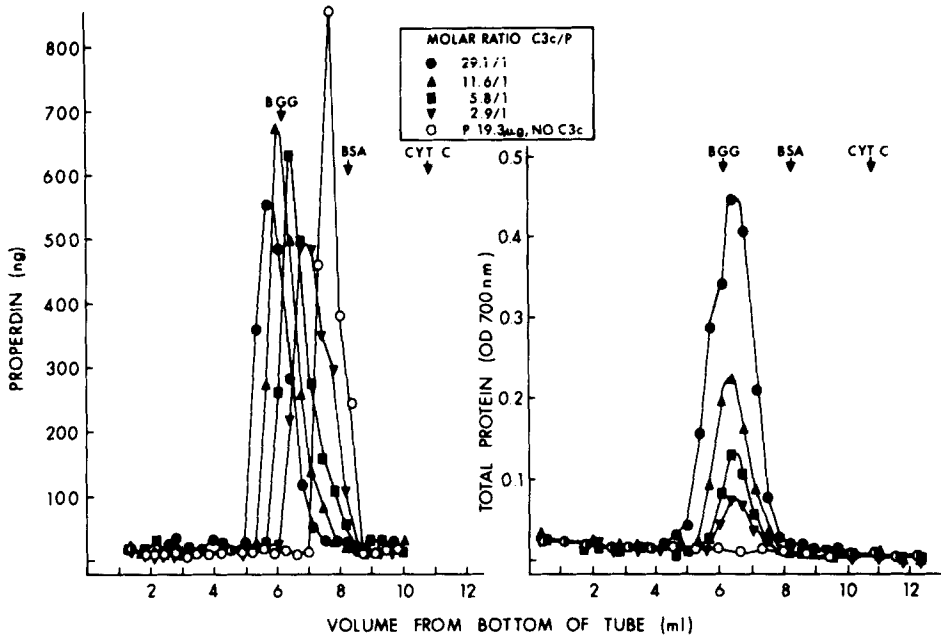

FIG. 8. The sedimentation of purified properdin in the presence or absence of various concentrations of purified C3c. Equal vol (0.5 ml) of properdin (control) or mixtures of properdin and C3c were centrifuged (41,000 rpm, 2°C, 17 h) on 1-15% gradients made in PBS. BGG, BSA, and CYT C served as markers (↓).

these experiments is suggested by the work of Cox (22) and will be discussed below.

A comparison of the molar ratios at which heavier sedimenting species of properdin were lost constituted one criterion to estimate the hierarchy of affinity constants between properdin and C3, C3b, and C3c. By this criterion, properdin demonstrated the strongest affinity for C3c; C3b was bound by properdin with slightly more affinity than was C3.

Sedimentation of Properdin in the Presence of C3 and Factor B. Although the data of the previous section clearly indicated that properdin interacts with C3 or C3 products, the more complex sedimentation behavior of properdin in normal human serum (Fig. 1) was not reproduced under the experimental conditions employed. It was recognized that more extensive experiments as a function of concentration of reactants and physicochemical parameters were required before any firm conclusion could be drawn concerning the possible participation of proteins other than properdin and C3 in species of properdin antigen heavier than 9S. Nevertheless it was of interest to examine the effect of factor B, a readily available purified protein of the alternative pathway, on the sedimentation of purified properdin and C3. In fact, as shown in Fig. 9, factor B was entirely without influence on the sedimentation of properdin-C3 mixtures, even though the concentration of factor B was at physiological level. This experiment also provided evidence that the interaction of properdin with C3 was not a general property of proteins other than C3. This conclusion is apparent

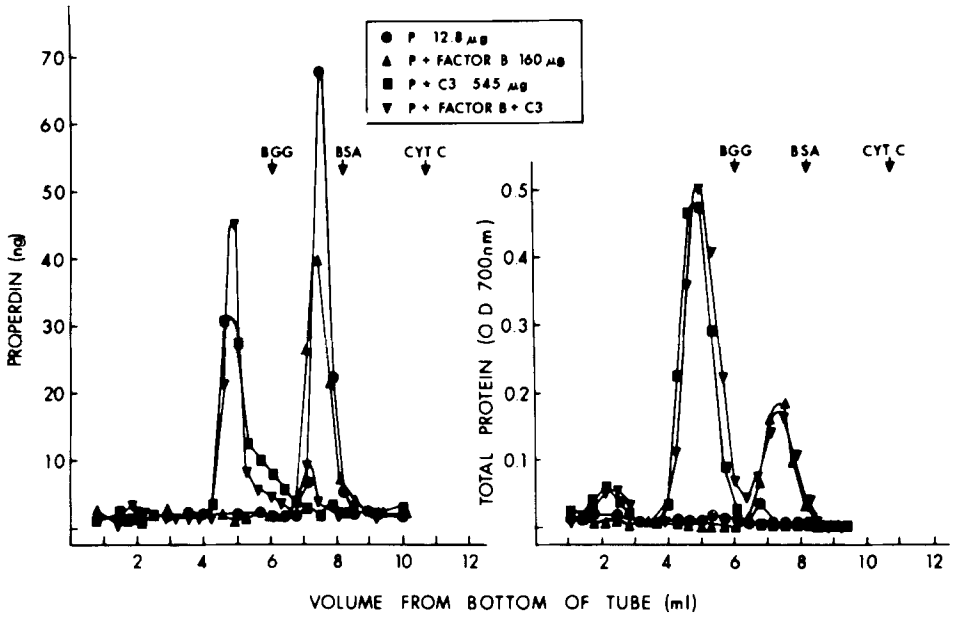

FIG. 9. Sedimentation of purified properdin alone or in the presence of factor B, C3, or factor B and C3. Equal vol of the indicated purified protein solutions (0.5 ml) were centrifuged (41,000 rpm, 2°C, 17.5 h) on 1-15% gradients made in PBS. BGG, BSA, and CYT C served as markers (↓).

from the data in Figs. 4 and 5 with C3-deficient human sera and from our observation that the sedimentation behavior of purified properdin was unaffected by the presence of high concentrations of BSA.

Discussion

Utilizing linear sucrose density gradient ultracentrifugation as an analytical tool, this paper has shown that "native" properdin (P) in either serum or plasma was separated into multiple sedimenting species (Fig. 1). For comparison, highly purified "activated" properdin ($\bar{P}$) sedimented as a single homogenous protein peak at ~6S. Throughout the course of these studies no properdin antigen was detected with sedimentation coefficients less than ~6S. Employing conditions of low ionic strength (0.075 μ) and cold temperature (2°C) species of properdin with Svedberg rates as high as 15S were found (Fig. 3). The experimental results may serve to explain, at least in part, the wide range of sedimentation rates (27S-5S) that have been earlier reported for partially purified preparations of properdin (1–5). These preparations of properdin, probably containing $\bar{P}$, may have been contaminated, for example, with sufficiently high concentrations of C3 or C3-degradative products to the extent that variable amounts of heavier sedimenting protein-protein complexes of C3 contaminants and properdin were present. Purified $\bar{P}$ complexed with purified C3, C3b, or C3c (Figs. 6–8). It is of interest that Minta (23) has reported that $\bar{P}$ added to normal serum or to serum rendered deficient in properdin sedimented heavier in sucrose gradients. He attributed the finding to aggregation of $\bar{P}$ or to complex formation of $\bar{P}$ with a

serum euglobulin (probably C3) mediated by an as yet incompletely characterized serum factor, factor F.

With dilution, the sedimentation of properdin in serum was altered by a shift toward lighter sedimenting species and the effect was reversed after reconcentration (Fig. 2). Previously it had been realized that the functional activity of the alternative pathway markedly decreased with dilution (1). Perhaps interaction of properdin with complexing proteins, of which at least one is native C3, constitutes a critical factor for the functional initiation and/or activity of properdin. Technically limited by the sample density that can be layered onto a sucrose gradient and the limitations of concentrating serum, heavier species of properdin were not demonstrated in concentrated serum relative to unconcentrated serum.

Increasing temperature from 2 to 23°C effected a shift of properdin to lighter species. It was not ascertained whether during the long gradient run at warm temperature the alternative pathway may have been activated, thereby accounting for the results obtained. It is important to emphasize that present data are not sufficient to describe the actual state of association of native properdin with other serum proteins in vivo nor to relate such associations to the functional activity of properdin in the alternative pathway.

Purified $\bar{P}$ mixed with purified C3 or C3 catabolic products sedimented in complex patterns that were dependent on the molar ratio of C3, C3b, or C3c to properdin (Figs. 6–8). Properdin-containing complexes exhibited the highest sedimentation coefficients at the highest molar ratio examined. Reduction of the molar ratio resulted in properdin species with smaller sedimentation coefficients; the smallest values were only slightly larger than control properdin. In some mixtures distinctly bimodal peaks sedimented intermediate between the heaviest and lighest properdin species. A plausible theoretical basis for these results is derived from the work of Cox which describes "the sedimentation and diffusion of macromolecular solutes that undergo rapidly re-equilibrating association-dissociation reactions of the type $nA \rightleftharpoons A_n$" (22). The sedimentation pattern generated is dependent on a variety of physicochemical parameters, including initial solute concentration and affinity constant, which dynamically influence the state of equilibrium of the system in an ultracentrifugal field. As a result, multiple boundaries or single boundaries of various shapes and at different positions arise. Although it is recognized that Cox's data relate to homogenous solute systems (22,24,25) they may nevertheless provide conceptual insight into the heterogeneous solute systems described in this paper ($\bar{P}$ and C3, $\bar{P}$ and C3b, or $\bar{P}$ and C3c).

Experiments are in progress that are aimed at distinguishing the possibilities that multiple sedimenting species of native properdin in serum are composed solely of properdin and C3 or whether additional serum factors are involved. Initial efforts are being made to pursue the studies already begun by incorporating into mixtures higher concentrations of purified $\bar{P}$ and C3 under various physicochemical conditions in an attempt to generate species of properdin heavier than the 9S complexes shown in Figs. 6 and 9. In addition mixtures of various combinations and concentrations of properdin, C3, factor B, and factor $\bar{D}$, another component of the alternative pathway, are being examined. In experi-

ments already completed, the presence of factor B in mixtures of purified $\bar{P}$ and C3 or $\bar{P}$ alone was without effect on the sedimentation of $\bar{P}$ (Fig. 9). In a preliminary experiment, the further addition of ~8,000 U of purified factor $\bar{D}$ (kindly supplied by Dr. Douglas T. Fearon, Robert B. Brigham Hospital, Boston, Mass.) to mixtures of $\bar{P}$, C3, and factor B at the concentrations shown in Fig. 9 resulted in appearance of a shoulder on the descending side of the 9S peak, corresponding to ~11S. Although this observation requires confirmation and extension, it suggests that complexes of properdin sedimenting more rapidly than 9S may in fact involve the further participation of constituents of the alternative pathway in addition to C3. In further experiments, one would choose ideally to study native or precursor proteins (26,27).

Macromolecular complexes of proteins are a recurrent finding in the classical C pathway. Protein-protein interactions characterize precursor molecules as well as molecules that subsequently become activated (28–36). Despite a less well-characterized sequence of reactions, several lines of evidence indicate that multimolecular complexes also assemble on the particulate surfaces of initiating agents of the alternative pathway. First, the cellular intermediate $EAC\overline{4,3,P}$ was shown to be 10- to 20-fold more susceptible to lysis compared with an analogous intermediate lacking properdin (37). For lysis the intermediates were incubated in the presence of factor B, factor $\bar{D}$, and a source of C3-9. The presence of properdin in $EAC\overline{4,3,P,B}$ probably stabilized the C3-convertase $\overline{C3b,B}$. Second, the zymosan intermediate ZX^{d2} contained C3b which provided a site for further interaction with factor B, thereby forming $ZX^{d2}\overline{C3b,B}$ (38). Circumstantial evidence supported the existence of the intermediate $ZX^{d2}\overline{C3b,P,B}$. Third, it has been shown that binding of radiolabeled $\bar{P}$ to zymosan required the presence of functionally active C3 (factor A) and factor B (39). Further, it was demonstrated that the washed intermediate complex ZX which was initially formed by incubating Z with serum was able to bind radiolabeled $\bar{P}$. Fourth, in a system utilizing agar gels as initiating agent, visible protein zones containing properdin and C3 but not factor B were formed after double diffusion of normal human serum and purified properdin. Factor B was required for the reaction (40). Finally, highly pertinent data were published while this paper was in final stages of preparation. Agglutination of the cellular intermediate $EAC\overline{4,3}$ by $\bar{P}$ was described in a reaction that was dependent on C3 but not C4 and which was competitively inhibited by soluble C3 or C3b. Further, formation of functionally active $\bar{P}$-C3 convertase, a C3-convertase, required $\bar{P}$, native C3, factors B and $\bar{D}$, and magnesium ions (41). Thus, multiple lines of evidence indicate that properdin complexes with other proteins of the alternative pathway but our communication is the first to our knowledge which demonstrates by physicochemical criteria the direct interaction of properdin and C3.

Complexes or aggregates of alternative pathway proteins in tissues, such as are demonstrated using immunofluorescent techniques, may occur by mechanisms other than active deposition via the alternative pathway sequence. We have demonstrated that purified $\bar{P}$ complexes with purified C3, C3b, and C3c and that the affinity of $\bar{P}$ for C3c may be higher than for C3 or C3b (Figs. 6–8). Consequently, properdin that is found deposited in tissue sections may, in fact,

be bound to C3b or C3c once these species are formed *pari passu* with the operation of other C-activating mechanisms. The finding of properdin deposits in various pathological situations should therefore not be interpreted as firm evidence for an initiating role for the alternative pathway in the absence of other supporting data.

Summary

Normal human serum subjected to sucrose density gradient analysis exhibited multiple sedimenting species of properdin antigen. Properdin antigen distribution was dependent on serum concentration, ionic strength, temperature, and the presence of C3, and was not dependent on the presence of divalent metal cations or blood coagulation. In mixtures of purified components, properdin sedimented heavier in the presence of C3, C3b, or C3c. Addition of factor B to mixtures containing C3 and properdin was without effect. These data provide insights into earlier discrepancies concerning the sedimentation behavior of partially purified properdin, indicate a propensity of some constituents of the alternative pathway to form protein-protein complexes, and suggest caution in interpretation of immunopathological studies in which properdin deposits are found in the presence of C3.

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