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Resolution and analysis of 'native' and 'activated' properdin

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A rapid and reproducible procedure for the resolution of 'native' and 'activated' forms of properdin (a component of the alternative activation pathway of complement), by gel filtration on the polyvinyl matrix Fractogel TSK HW-55(S), is reported. This fractionation permitted effective screening of samples for conditions that cause activation. Only 'native' properdin was detected in serum, even after activation of the alternative pathway by yeast cell walls. Transformation of 'native' into 'activated' properdin *in vitro* was produced by freeze–thawing of the protein, but not upon binding to and dissociation from the C3 convertase, C3bBb. Electron microscopy showed that only the 'native' population contained the discrete cyclic structures described previously by Smith, Pangburn, Vogel & Müller-Eberhard [(1984) J. Biol. Chem. **259**, 4582–4588]. 'Activated' properdin, which was eluted from the gel-filtration column close to the breakthrough peak, was mainly composed of large amorphous aggregates. We therefore conclude that properdin 'activation' is not a physiological event that occurs in serum on complement activation, but is an artifact of isolation. Fractionation of properdin on Fractogel TSK HW-55(S) has, however, enabled detailed analysis of functional heterogeneity within the 'native' population.

INTRODUCTION

The alternative pathway of complement constitutes a non-adaptive system of early defence against host invasion by bacteria and other pathogenic organisms (Pillemer *et al.*, 1954; Pillemer, 1956; Müller-Eberhard & Schreiber, 1980). Central to activation is the generation of the C3 convertase, C3bBb, by the interaction of the components C3b, Factor B and Factor D. Properdin amplifies activation by binding to the C3 convertase and stabilizing it against intrinsic decay of Bb from the complex (Fearon & Austen, 1975). The C3bBbP complex may also be more resistant than C3bBb to specific inactivation events mediated by the regulatory components, Factors I and H (Medicus *et al.*, 1976*a*).

Early isolation procedures for properdin utilized specific adsorption on and release from zymosan, a polysaccharide activator of the alternative pathway found in yeast cell walls; from these a high-molecular-mass protein (27 S) was recovered (Pillemer *et al.*, 1954). Later work suggested the presence of a smaller (7 S) form of properdin (Pondman *et al.*, 1960), but it was not until zymosan adsorption was omitted from isolation procedures that the bulk of the properdin was isolated as a 5–7 S protein (Rothstein, 1962; Pensky *et al.*, 1968).

Early studies also demonstrated an alteration in the electrophoretic mobility of properdin in aged or alternative-pathway-activated sera (McLean & Michael, 1972; Minta, 1975). This change paralleled an alteration in the mobility of C3, but the nature of the altered-mobility species was not fully resolved (McLean *et al.*, 1975; Adam *et al.*, 1975). Furthermore, properdin eluted from zymosan was claimed to have an altered (β) mobility and not the γ mobility of properdin in freshly drawn serum (Adam *et al.*, 1975).

In addition to alterations in electrophoretic mobility, functional differences between different preparations of properdin were observed. Some bound to EAC3b at physiological ionic strength and induced C3 consumption when added to serum, whereas others had neither of these properties (Pillemer *et al.*, 1954; Lepow *et al.*, 1959; Todd *et al.*, 1959; Pondman *et al.*, 1960; Götze & Müller-Eberhard, 1974; Fearon *et al.*, 1974; Medicus *et al.*, 1976*a*; Götze *et al.*, 1977). Clearly, properdin in unfractionated serum does not initiate C3 consumption. Thus those preparations that activated C3 when added back to serum and that bound to C3b at physiological ionic strength were termed 'activated', and those that did neither were termed 'precursor' properdin. As both forms stabilized surface-bound C3bBb (Medicus *et al.*, 1976*a*), these findings led to the concept of properdin 'activation' by association with a C3 convertase (Götze & Müller-Eberhard, 1974; Medicus *et al.*, 1976*a*,*b*; Götze *et al.*, 1977). Evidence for both antigenic differences between 'activated' and 'precursor' properdin (Minta, 1975, 1976*b*) and proteolytic cleavage of the 'precursor' in the generation of the 'activated' protein (Minta, 1976*a*; Minta & Kunar, 1976) were subsequently claimed. However, on more detailed analysis these differences could not be substantiated (Götze *et al.*, 1977; Medicus *et al.*, 1980; Reid & Gagnon, 1981), and it was suggested that 'activation' involved a conformational change in the protein, induced by association with a C3 convertase (Medicus *et al.*, 1976*b*, 1980; Götze *et al.*, 1977). Since the 'precursor' form could be recovered by elution from a C3 convertase, the change was assumed

Abbreviations used: PBS, phosphate-buffered saline; CFD, complement fixation diluent; C4, complement component C4; C3b and Bb, the larger activation fragments of the complement components C3 and Factor B respectively; P, properdin; EAC3b, sheep erythrocytes coated with antibody and C3b; yeast-C3b, yeast cell walls coated with C3b.

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to be at least partially reversible. Interconversion of the two forms *in vitro* by repeated freezing and thawing (generating 'activated' properdin) and by mild denaturation (regenerating 'precursor' or 'native' properdin) supported the idea of a conformational change in the generation of 'activated' properdin (Medicus *et al.*, 1980). However, most preparations still contain a proportion of the 'activated' form, in which an undefined structural transformation is presumed to have occurred.

Detailed structural analysis of properdin has shown that it is composed of apparently identical subunits of molecular mass about 50000 Da (Minta & Lepow, 1974; Minta, 1976; Reid & Gagnon, 1981; DiScipio, 1982). The number of subunits contained in the 5–7 S properdin oligomer has been extensively investigated. Diffusion and ultracentrifugation measurements indicate a highly asymmetric molecule, with a molecular mass of about 200000 Da (Pensky *et al.*, 1968; Minta & Lepow, 1974; DiScipio, 1982), and have been interpreted as evidence for both trimer (DiScipio, 1982) and tetramer (Minta & Lepow, 1974) association of subunits. Electron micrographs have, however, shown a heterogeneity of association, with dimers (30%), trimers (45%), tetramers (10%) and higher oligomers (16%) all being present (Smith *et al.*, 1984).

Much of the difficulty in defining differences between 'activated' and 'native' properdin has arisen because it has not been possible to separate the two forms with subsequent recovery of 'activated' properdin. For example, although 'activated' properdin can be removed by passage of a mixture of both forms through C3b–Sepharose, it cannot be eluted with either chaotropic salts or denaturants (Medicus *et al.*, 1980). In the present paper we describe a simple and rapid method for the fractionation of 'native' and 'activated' Properdin, with recovery of both forms. This procedure has enabled clarification of the differences between them and also definition of the conditions under which interconversion does, and does not, occur.

MATERIALS AND METHODS

Buffers and reagents

PBS is 9.6 mM-KH_2PO_4/NaOH buffer, pH 7.2, containing 0.14 M-NaCl. CFD is 4 mM-sodium barbitone buffer, pH 7.2, containing 0.145 M-NaCl, 0.83 mM-$MgCl_2$ and 0.25 mM-$CaCl_2$, and is prepared from Complement Fixation Test Diluent tablets (Oxoid). CFD dextrose is CFD diluted 1:1 in 5% (w/v) D-glucose.

Gelatin, di-n-butyl phthalate, dinonyl phthalate and Tween 20 were purchased from BDH Chemicals. DEAE-Sephacel, CM-Sephadex C-50, bovine serum albumin and myoglobin (sperm whale) were obtained from Sigma Chemical Co. Activated-thiol–Sepharose 4B, Sepharose CL-4B, Sepharose 6B, DEAE-Sephadex A-50, Sephadex G-25 (medium grade), Sephadex G-75 (superfine grade), Sephadex G-150 (superfine grade), Sephadex G-200 and Blue Dextran 2000 were all products of Pharmacia Fine Chemicals. Hydroxyapatite (HTP grade) was purchased from Bio-Rad Laboratories, and Fractogel TSK HW-55(S) was obtained from Merck. Iodogen was the product of Pierce Chemical Co., and $Na^{125}I$ (carrier-free) was supplied by Amersham International.

Yeast cell-wall suspensions were prepared in accordance with Harrison & Lachmann (1986). Lysine–Sepharose 4B was prepared by using the CNBr procedure of March *et al.* (1974).

Complement components

Complement components C3, properdin and Factors B and D were isolated from a single fractionation of 4 litres of human plasma. The procedures used, with the exception of the modifications noted below, are described elsewhere (Harrison & Lachmann, 1986).

Properdin was isolated by chromatography on DEAE-Sephacel followed by CM-Sephadex C-50 (400 ml column; 2-litre linear gradient from 20 mM-KH_2PO_4/NaOH bufffer, pH 6.0, containing 0.15 M-NaCl to 20 mM-KH_2PO_4/NaOH buffer, pH 6.0, containing 0.3 M-NaCl) and gel filtration on Sephadex G-200 (95 cm × 2.5 cm column) in 20 mM-KH_2PO_4/NaOH buffer, pH 7.2, containing 0.5 M-NaCl and 5 mM-NaN_3. The purity of this preparation is illustrated in Fig. 1. C3 was isolated by sequential chromatography on DEAE-Sephacel, Sepharose 6B and DEAE-Sephadex A-50. The C3 pool from DEAE-Sephadex was used in the preparation of EAC3b and yeast-C3b. C3 used in all other experiments was further purified on hydroxyapatite. Factor B was isolated by chromatography on DEAE-Sephacel, Sepharose 6B, CM-Sephadex C-50 and hydroxyapatite. Factor D was isolated by chromato-

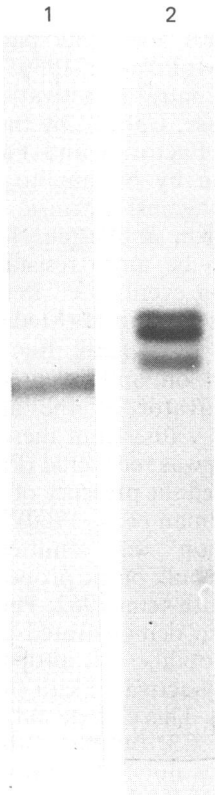

Fig. 1. SDS/polyacrylamide-gel electrophoresis analysis of purified properdin

Samples (10 μg, track 1; 15 μg, track 2) were denatured by heating at 100 °C for 2 min in 1% SDS in the presence (track 1) or in the absence (track 2) of 1% (v/v) 2-mercaptoethanol. A 9% polyacrylamide gel and the buffer system of Laemmli (1970) were used, and the gel was stained with Coomassie Blue R250.

graphy on DEAE-Sephacel followed by successive gel-filtration steps on Sephadex G-150 (superfine grade) and Sephadex G-75 (superfine grade).

Radiolabelling of proteins

Proteins were radiolabelled at 4 °C with ^{125}I by the solid-phase Iodogen method (Fraker & Speck, 1978). Unbound ^{125}I was removed either by gel filtration on Sephadex G-25 (medium grade) or, for properdin, by exhaustive dialysis against PBS. C3i (inactive C3) was removed from C3 samples, immediately before radiolabelling, by reaction with activated-thiol–Sepharose (Tack *et al.*, 1980). Labelled proteins were generally stored frozen in 1% (w/v) bovine serum albumin at −70 °C. Specific radioactivities ranged from 0.2 to 1 μCi/μg. ^{125}I-labelled 'native' properdin was obtained by gel filtration of ^{125}I-labelled unfractionated properdin, as shown in Fig. 4. Only fractions from the centre of the peak of 'native' properdin were used; these were stored at 4 °C.

Cellular intermediates and binding studies

EAC3b. EAC3b were made by using sheep erythrocytes (E), rat monoclonal IgM anti-E (SO16), yeast-treated guinea-pig serum ('R3') and partially purified C3, as described previously (Harrison & Lachmann, 1986). Cellular intermediates were calibrated by measuring the uptake of ^{125}I-labelled monoclonal antibodies specific for C3 and its fragments (Lachmann *et al.*, 1980), and generally carried 10000–50000 C3b molecules/cell.

Binding studies on cell intermediates were performed with radiolabelled components. Components and cell intermediates were incubated for 15–30 min at 37 °C to allow equilibration between bound and unbound protein. Samples (50–100 μl) were then layered (in duplicate or triplicate) on to 200 μl of an oil mixture containing di-n-butyl phthalate and dinonyl phthalate [4:1, v/v, prewarmed to 37 °C in 400 μl Microfuge tubes (Cuatrecasas & Hollenberg, 1976; Iida *et al.*, 1982)]. These were centrifuged at 10000 ***g*** for 1 min and then rapidly frozen. The pellets, containing the cell intermediates, were cut off, and the distribution of the component was assessed by counting the amount of radioactivity in both the supernatants and the pellets.

Yeast-C3b. Yeast cell walls were coated with C3b as follows. Washed cell walls were resuspended at 12% (v/v) in CFD. Normal human serum was added to 5% (v/v), and the mixture was stirred for 40 min at 37 °C. The cell walls (containing some bound C3b) were then washed three times in PBS and once in CFD before resuspension, at 20% (v/v), in CFD containing 0.2% (w/v) bovine serum albumin and 0.15 mM-$NiCl_2$. This suspension was incubated at 37 °C with Factor B (40 μg/ml) and Factor D (1 μg/ml) for 5 min to generate a surface-bound C3 convertase. After a washing, the cell walls were further incubated at 37 °C for 20 min in the same volume of CFD containing 0.2% bovine serum albumin and containing C3 (1 mg/ml) to amplify C3b deposition. The yeast-C3b were finally washed in PBS, incubated for 30 min at 37 °C in PBS containing 10 mM-EDTA and 0.5 M-NaCl, and then washed twice in the same buffer (to remove properdin and Factor B) and once in PBS. Binding studies with these yeast-C3b were performed as described above for EAC3b. About 1 pmol of C3b was fixed per mg of cell walls.

Gel filtration on Fractogel TSK HW-55(S)

An 89 ml column (113 cm × 1 cm) of Fractogel TSK HW-55(S) was used for all fractionations of 'native' and 'activated' properdin. These were performed in 20 mM-KH_2PO_4/NaOH buffer, pH 7.0, containing 0.2 M-NaCl, at a flow rate of 20 ml/h. The eluate was collected in 1.3 ml fractions. Samples were loaded in buffer containing 20% (w/v) sucrose and either 1 mg of gelatin/ml or 20% (v/v) of a lipoprotein-enriched plasma fraction (devoid of complement proteins). Gelatin and the plasma fraction enhanced recovery of the loaded protein to the same extent. Myoglobin, at 2.5 mg/ml and monitored at 400 nm, was included to provide a reference elution position. For studies by electron microscopy, the column was pre-run with 0.5 ml of PBS containing 4 mg of gelatin/ml and 20% (v/v) of the lipoprotein-enriched plasma fraction immediately before chromatography of purified properdin, which was loaded in 20% sucrose. In this case, fractions were collected in chromic acid-washed tubes.

Haemolytic assays for properdin

Haemolytic assays for 'activated' and 'activated' plus 'native' properdin were performed by using adaptations of the methods of Medicus *et al.* (1980). A stock B/D solution of Factor B (40 μg/ml) and Factor D (0.5 μg/ml) in CFD containing gelatin (1 mg/ml) and 5 mM-NaN_3 (CFD/gel/azide) was made. Portions (20 μl) of dilutions of properdin samples were diluted in CFD and added to either 20 μl of CFD/gel/azide ('activated' properdin assay) or 20 μl of the B/D solution ('activated' + 'native' properdin assay). These were incubated with 20 μl of 5% (v/v) EAC3b in CFD/gel/azide for 5 min at 37 °C. The reaction was stopped by the addition of 1 ml of PBS at 4 °C. The cells were rapidly pelleted by centrifugation, washed with 1 ml of CFD (chilled to 4 °C) and then resuspended, at 37 °C, in 40 μl of a 1:1 dilution, again in CFD/gel/azide, of the stock B/D solution. After 5 min incubation, 40 μl of CFD containing 20 mM-EDTA was added. The cells were incubated for a further 10 min to permit decay of non-properdin-stabilized C3bBb convertases, and then 1 ml of 1% (v/v) normal guinea-pig serum in CFD containing 10 mM-EDTA was added. After a further incubation (10 min at 37 °C) intact cells were pelleted by centrifugation and the degree of lysis was determined from the absorbance of the supernatants at 415 nm.

C3 conversion in normal human serum

A 1 ml portion of normal human serum was supplemented with $MgCl_2$ (0.33 mM) and the pH adjusted to 6.5. Then 20 μCi of ^{125}I-labelled C3 was added, and 50 μl of this serum reagent was incubated with 25 μl of each properdin sample at 37 °C for 1 h. Complement activity was stopped by the addition of 200 μl of 75 mM-Tris/HCl buffer, pH 6.5, containing 1% (w/v) SDS, 1% (v/v) 2-mercaptoethanol and 10% (v/v) glycerol. A 5 μl portion of each sample was analysed by SDS/polyacrylamide-gel electrophoresis with the buffer system of Laemmli (1970) and a 10% polyacrylamide gel. After electrophoresis, the gel was fixed, dried and autoradiographed, and the extent of C3 conversion was determined by excision and counting of the radioactivity of the C3-derived bands.

Agglutinin of EAC3b by properdin (see Schreiber *et al.*, 1975)

Samples containing properdin were serially diluted in 20 μl of CFD in plastic micro-titre plates. Then 40 μl of CFD/gel/azide containing 5×10^6 EAC3b cells was added to each well, the plates were agitated, and the cells were allowed to settle at room temperature until the limiting dilution for agglutination could be recorded.

Antigenic assay of properdin

Antisera to human properdin were raised in New Zealand White rabbits, and the IgG fraction of the antiserum was prepared, by the method of Steinbuch & Audran (1969), from serum taken 37–48 days after the initial immunization. An indirect radioimmunoassay, modified from the method of Minta *et al.* (1973), was used to quantify non-radiolabelled properdin. Flexible U-bottomed plastic micro-titre plates were coated (overnight at 4 °C) with rabbit anti-(human properdin) antibody (50 μl of IgG, at 20 μg/ml, in 0.1 M-$NaHCO_3$, pH 9.5, per well). The antibody was then removed and the plates were washed six times with PBS containing 5 mM-NaN_3, 0.05% (v/v) Tween 20 and bovine serum albumin (2 mg/ml). Serial dilutions of properdin samples, in 100 μl of the wash buffer, were incubated (overnight at 4 °C) in the antibody-coated wells with 10000 c.p.m. (10–20 ng) of ^{125}I-labelled properdin. The plates were finally washed a further six times, and the wells were excised and their radioactivities counted. The assay was calibrated against dilutions of normal human serum.

RESULTS

Analysis of properdin size heterogeneity by gel filtration on Fractogel TSK HW-55(S)

Fig. 2 shows the elution profile given by gel filtration of ^{125}I-labelled properdin on Fractogel TSK HW-55(S). The position of the major peak (Fig. 2*b*, fraction 33) corresponds to an apparent molecular-mass range, for a globular protein, of 400–2000 kDa, centred at about 800 kDa. Rechromatography of portions of individual fractions (Fig. 2*c*) suggested that this broad peak (fractions 30–36) contained at least three discrete components. A smaller peak, centred on fraction 26 in Fig. 2(*b*), which was eluted at, or near, the void volume, also maintained its elution position on rechromatography. All fractions produced identical band patterns when analysed by SDS/polyacrylamide-gel electrophoresis under either reducing (single band) or non-reducing (two or three closely spaced bands) conditions (see Fig. 1).

Resolution of 'native' and 'activated' properdin

A sample of properdin (0.5 mg), which contained both 'native' and 'activated' forms, was chromatographed on Fractogel TSK HW-55(S). The eluate was monitored by radioimmunoassay (Fig. 3*a*) and assayed for the various activities of 'native' and 'activated' properdin (Figs. 3*b*, 3*c* and 3*d*). The elution profile was similar to that given by radiolabelled properdin. (The apparent separation, within the main peak, of two species of purified properdin is not normally observed, and should be treated with circumspection as it is based on the amount of properdin found for a single fraction.) Only material

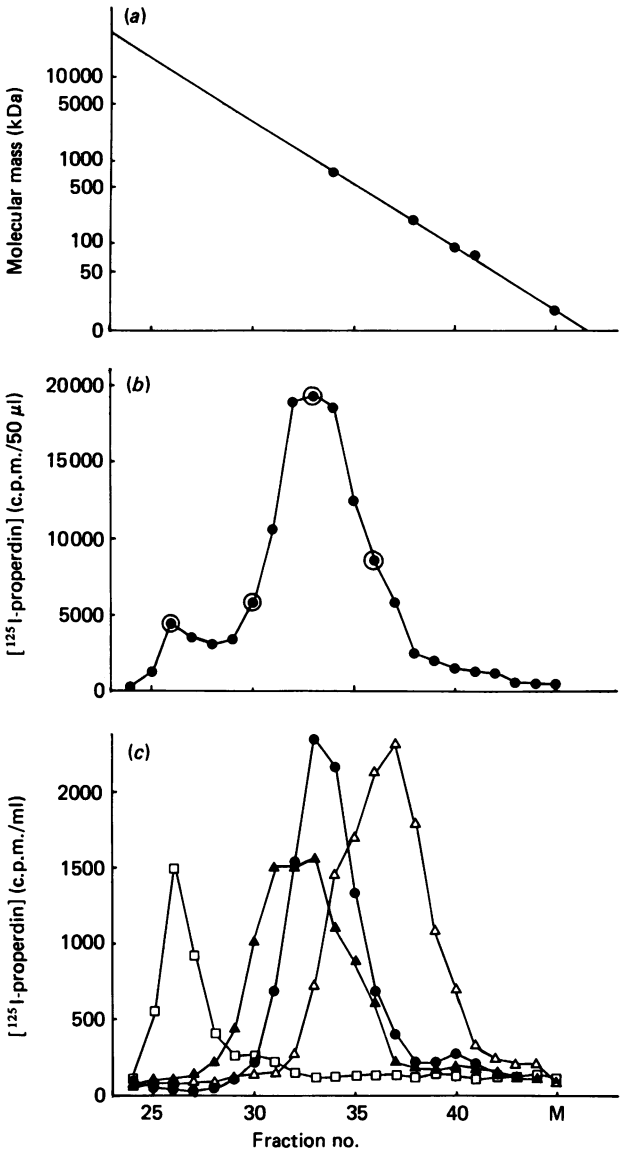

Fig. 2. Elution profile of ^{125}I-labelled properdin on Fractogel TSK HW-55(S)

(*a*) Molecular-mass calibration of elution volumes from Fractogel TSK HW-55(S). A 0.5 ml portion of a mixture containing α_2-macroglobulin (725 kDa) (3 mg/ml), C4 (205 kDa) (1 mg/ml), Factor B (93 kDa) (0.84 mg/ml), bovine serum albumin (68 kDa) (6 mg/ml) and myoglobin (17.5 kDa) (2.5 mg/ml) was chromatographed on Fractogel TSK HW-55(S) as described in the text. The elution position of each protein is indicated. (*b*) Elution profile of radiolabelled properdin on Fractogel TSK HW-55(S). A 3.5 μCi portion of ^{125}I-labelled properdin in 0.265 ml of PBS containing gelatin (2 mg/ml), 20% (w/v) sucrose and myoglobin (5 mg/ml) was chromatographed on Fractogel TSK HW-55(S), and samples of each fraction were taken for counting of radioactivity. Portions of single fractions (ringed in the elution profile shown) were further analysed by re-chromatography. (*c*) Re-fractionation of properdin fractions. Gelatin (1 mg/ml), myoglobin (5 mg/ml) and sucrose (20%, w/v) were added to 0.4 ml portions of fractions 26 (□), 30 (▲) and 36 (△), and to a 0.1 ml portion of fraction 33 (●). These were then re-fractionated on Fractogel TSK HW-55(S), producing the elution profiles shown. M indicates the elution position of myoglobin.

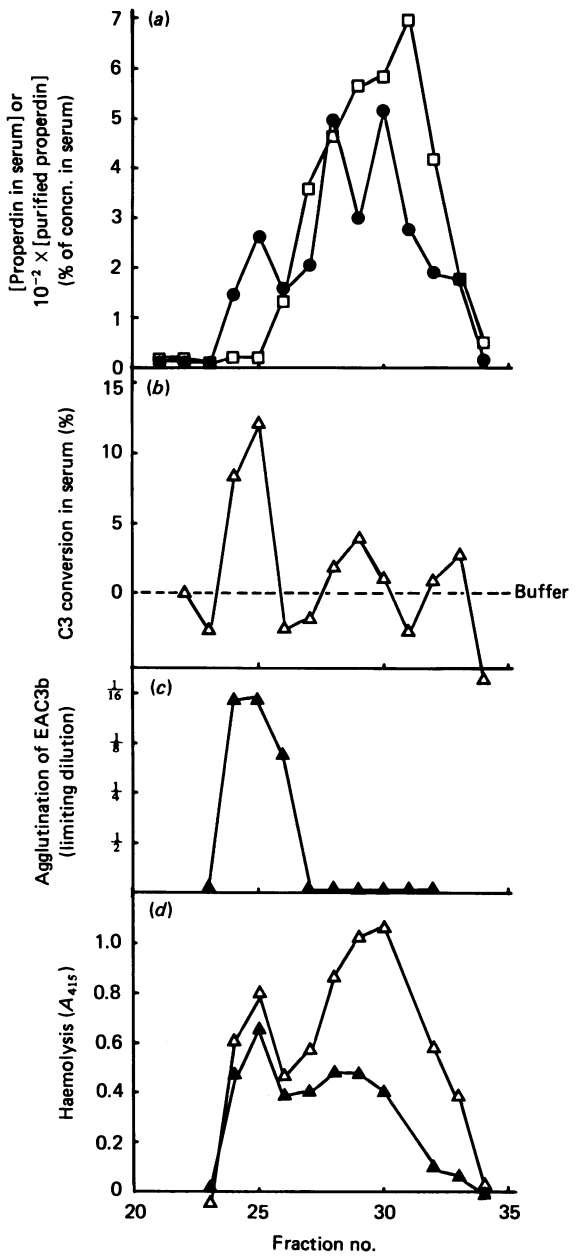

Fig. 3. Resolution of 'native' and 'activated' properdin

(*a*) Elution profiles of purified properdin and of properdin in normal human serum chromatographed on Fractogel TSK HW-55(S). A 0.4 mg portion of purified properdin in PBS (●) and a 0.4 ml portion of normal human serum (□) were separately fractionated on Fractogel TSK HW-55(S), and the eluates were assayed for properdin by the indirect radioimmunoassay procedure as described in the text to produce the elution profiles shown. (*b*) Activation of the alternative pathway by properdin. Portions of the above fractions of gel-filtered purified properdin were screened for ability to cause C3 conversion in whole serum as described in the text. (*c*) Agglutination of EAC3b. Serial dilutions of fractions were tested for ability to cause agglutination of EAC3b as described in the text. The limiting dilutions that caused significant agglutination are shown. (*d*) haemolytic properties. Fractions were assayed for 'activated' properdin (△) and for 'activated' plus 'native' properdin (▲) by using the haemolytic assays described in the text.

contained in the early minor peak agglutinated EAC3b (Fig. 3*c*) or initiated greater than 5% C3 conversion in normal human serum (Fig. 3*b*). Fig. 3(*d*) shows that the haemolytic activity of the major, but not the minor, properdin peak was enhanced with concurrent rather than sequential incubation of EAC3b with properdin and Factor B plus Factor D. These results indicate that the early minor peak is 'activated' properdin and that the major peak is 'native' properdin.

A similar fractionation of normal human serum was also monitored, by radioimmunoassay, for properdin. The distribution of antigen was similar to that of the major peak of purified properdin except that there was no early peak corresponding to the 'activated' form (Fig. 3*a*).

Binding properties of 'native' and 'activated' properdin

Binding of properdin to surface-fixed C3b is most directly assessed by using ^{125}I-labelled protein. Fractionation of ^{125}I-labelled properdin and subsequent assay of fractions for binding to EAC3b, EAC3bB and EAC3bBb are shown in Figs. 4(*a*) and 4(*b*). Only the earliest fractions bound to EAC3b or, more avidly, to EAC3bB. All major and minor peak fractions contained properdin that bound to EAC3bBb, although the efficiency of uptake diminished steadily towards the later fractions. The recovered material consistently contained slightly less 'activated' properdin than the loaded material, implying that some 'activated' properdin was still trapped on the column despite blocking with gelatin.

Fig. 4(*c*) shows analysis of binding to yeast-C3b. Again, only the early peak fractions bound at physiological ionic strength. At low ionic strength the binding profile was altered, with later fractions binding more avidly under these conditions. These results are consistent with the findings of DiScipio (1981) on the binding of unfractionated properdin to C3b-coated zymosan.

Finally, re-addition of portions of the resolved radioactive fractions to portions of normal human serum showed that the earlier-eluted components are preferentially fixed during activation of the alternative pathway by limiting amounts of yeast cell walls (Fig. 4*d*). All of the bound properdin was eluted in CFD containing 10 mM-EDTA and 0.65 M-NaCl.

Interconversion of 'activated' and 'native' forms of properdin

The above results show that properdin, chromatographed on Fractogel TSK HW-55(S), is separated into its 'activated' and 'native' forms. The same system was therefore used to investigate interconversion between them. Firstly, samples of ^{125}I-labelled properdin, rich in the 'activated' form, were treated with various concentrations of guanidinium chloride, as described by Medicus *et al.* (1980). After dialysis to remove guanidine the ability of properdin samples, treated with increasing concentrations of denaturant, to bind to EAC3b, EAC3bB and EAC3bBb were compared with the distribution between 'activated' and 'native' peaks of the same samples after chromatography on Fractogel TSK HW-55(S). The results of this experiment are shown in Table 1(*a*). Guanidinium chloride produced a dose-dependent loss of the early 'activated' peak, without significant changes in the position or shape of the main 'native' peak (a small amount of radiolabel was associated with low-molecular-mass material at the

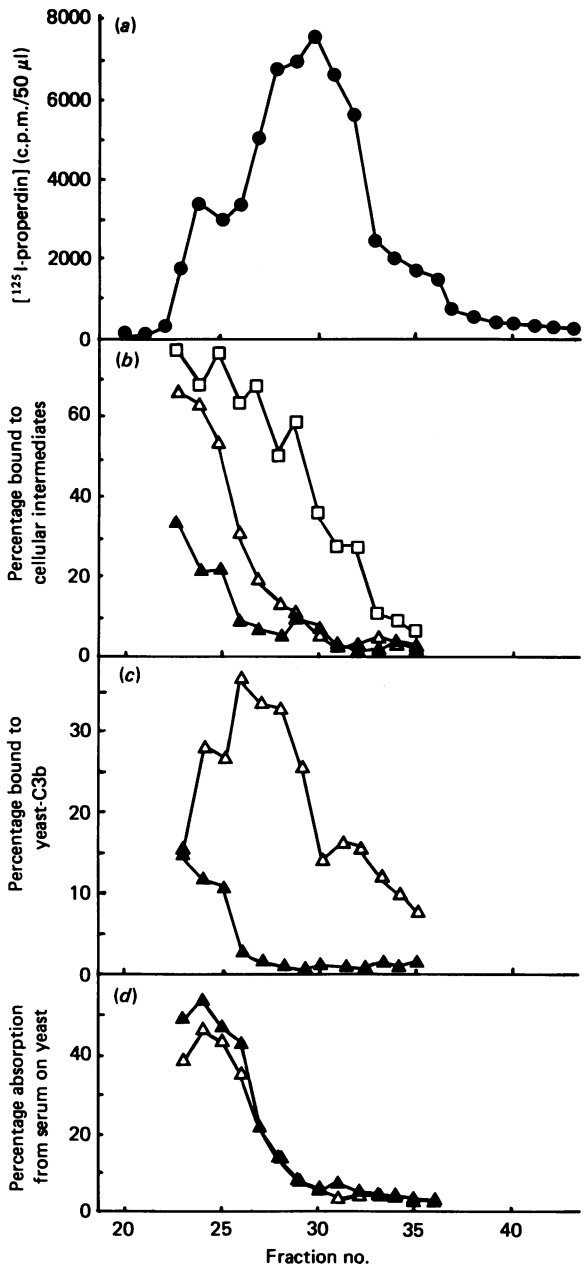

Fig. 4. Binding properties of 'native' and 'activated' properdin

(*a*) Radioactivity elution profile obtained from fractionation of 1 μCi of ^{125}I-labelled properdin (1.5 μg) on Fractogel TSK HW-55(S). (*b*) Binding of fractions to EAC3b, EAC3bB and EAC3bBb. A 50 μl portion of each fraction (diluted in column buffer to 3000 c.p.m./50 μl) was incubated at 37 °C for 20 min with 0.15 ml of CFD containing 0.2 mM-$NiCl_2$, bovine serum albumin (4 mg/ml) and 3.75×10^7 EAC3b cells, and either buffer alone (EAC3b, ▲), or Factor B (50 μg/ml) (EAC3bB, △), or Factor B (20 μg/ml) plus Factor D (0.25 μg/ml) (EAC3bBb, □). Duplicate 0.1 ml samples were assayed for binding as described in the text. (*c*) Binding of fractions to C3b-coated yeast. Samples were assayed for binding to yeast-C3b at 0.75% (v/v) in either PBS containing bovine serum albumin (3 mg/ml) (▲) or PBS/3 containing bovine serum albumin (3 mg/ml) (△). The particles were again incubated for 20 min at 37 °C before uptake was assessed as described in the text. (*d*) Recovery of properdin fractions from serum by adsorption with yeast cell walls. A 50 μl portion of each fraction was mixed with a 0.45 ml portion of normal human serum at 15 °C. Then 50 μl of a 2% (v/v) yeast cell-wall suspension (in CFD) was added to each. After 1 h at 15 °C, the yeast was recovered by centrifugation, washed once with 0.6 ml of PBS, then incubated for 1 h at 37 °C with 0.5 ml of CFD containing 10 mM-EDTA and 0.65 M-NaCl. The yeast was again removed by centrifugation, and the radioactivities of this residue and all the supernatants were counted to obtain the proportions of properdin initially bound in normal human serum (▲) and subsequently eluted in EDTA (△).

highest guanidine concentration used). This loss of the early peak was accompanied by a loss of avidity of the sample for EAC3b, but binding to EAC3bBb was retained.

Repeated freezing and thawing (freeze–thaw) has long been associated with the conversion of 'native' into 'activated' properdin. Therefore samples from the peak fractions of the 'native' peak given by gel filtration of ^{125}I-labelled properdin on Fractogel TSK HW-55(S) were added to portions of unfractionated properdin. These were subjected to five cycles of freeze–thaw (−78 °C and 37 °C) and then compared by analysis on Fractogel TSK HW-55(S). The results, shown in Table 1(*b*), demonstrated that the freeze–thaw process does promote 'activation', especially at properdin concentrations at or exceeding 20 μg/ml.

Investigation of the properties of properdin eluted from EAC3bBb

A sample of 'native' ^{125}I-labelled properdin, which did not bind to EAC3b, was incubated with EAC3b plus Mg^{2+} and Factors B and D. [The peak fraction of 'native' ^{125}I-labelled properdin chromatographed on Fractogel TSK HW-55(S) was used as this gives a sharper secondary fractionation (see Fig. 2) and thus greater sensitivity to appearance of 'activated' species.] After washing of the EAC3bBbP complex with PBS, 97.3% of the bound radiolabel was eluted in PBS containing 10 mM-EDTA (Table 2*a*). Furthermore, re-fractionation of the eluted material on Fractogel TSK HW-55(S) showed that no alteration in distribution had occurred (Fig. 5). The absence of activation was confirmed by direct binding studies on the cell intermediates EAC3b, EAC3bB and EAC3bBb (Table 2*b*); the eluate retained the binding properties of 'native' properdin.

Investigation of the properties of 'native' properdin after addition to serum and incubation with yeast cell walls

A portion of 'native' ^{125}I-labelled properdin [again from a peak fraction of the 'native' peak of ^{125}I-labelled properdin fractionated on Fractogel TSK HW-55(S)] was added to freshly drawn serum, and the serum was incubated with yeast cell walls (15%, v/v) at 17 °C (1 h) to activate the alternative pathway (mimicking the initial zymosan adsorption conditions of Pillemer *et al.*, 1954). The yeast cell walls were then washed with CFD, and bound properdin was eluted by incubation for 135 min at 37 °C in CFD containing 10 mM-EDTA, 0.5 M-NaCl and bovine serum albumin (7 mg/ml). The eluted properdin was chromatographed on Fractogel TSK HW-55(S). As with properdin binding to and elution

Table 1. Correlation of gel-filtration distribution of properdin with transitions between 'activated' and 'native' forms

(*a*) Transition produced by incubation in guanidinium chloride. Portions (0.6 μCi; 0.5 μg) of ^{125}I-labelled properdin (an unfractionated mixture of 'activated' and 'native') were incubated (75 min at 22 °C) in 0.5 ml of PBS containing bovine serum albumin (2 mg/ml) BSA and various concentrations of guanidinium chloride. The samples were then separately dialysed, at 4 °C, against PBS (four changes of 50 ml each). Gelatin (1.5 mg/ml), myoglobin (5 mg/ml) and sucrose (20%, w/v) were then added to 0.2 ml portions of each, and the samples were chromatographed on Fractogel TSK HW-55(S). The percentages of radioactivity eluted in the first peak were determined from the resulting elution profiles. Portions (20 μl) of the dialysed samples were also assayed for binding to EAC3b, EAC3bB and EAC3bBb in the conditions described in the legend to Fig. 4. (*b*) Transition produced by cycles of freezing and thawing. Samples (50 μl) of ^{125}I-labelled 'native' properdin (12 nCi; 10 ng), purified by filtration on Fractogel TSK HW-55(S), were mixed with 50 μl of dilutions of unfractionated purified properdin, and diluted, in PBS, to the final concentrations shown. These samples were subjected to five cycles of freeze–thaw (−78 °C and 37 °C) and then analysed by chromatography on Fractogel TSK HW-55(S) as described above in (*a*).

(*a*) Transition produced by incubation in guanidinium chloride

[Guanidine] (M)	Percentage binding to: EAC3b	EAC3bB	EAC3bBb	Peak I (%)
0	49.8	59.1	60.1	11.3
0.6	35.8	65.0	71.5	5.0
1.0	24.5	60.6	70.6	4.6
1.8	19.6	54.8	67.3	4.2

(*b*) Transition produced by cycles of freezing and thawing

[Properdin] (μg/ml)	No. of cycles of freeze–thaw	Peak 1 (% of label)
0.1	0	0.8
0.1	5	1.1
20	5	10.3
100	5	21.8
500	5	13.2

from EC3bBb (Fig. 5), there was no alteration in gel-filtration profile and hence no evidence for activation of properdin.

Investigation of the properties of unfractionated properdin after addition to serum and incubation with yeast cell walls

The above experiments indicate that there is no activation of 'native' properdin on binding to and elution from a surface-bound C3bBb convertase. However, as 'activation' of properdin has been associated with extraction from serum by zymosan adsorption

Table 2. Interaction of 'native' properdin with EAC3bBb

(*a*) Binding to, and elution from, EAC3bBb. Portions (0.15 μCi; 0.2 ml) of ^{125}I-labelled 'native' properdin [obtained by chromatography on Fractogel TSK HW-55(S)] were mixed with bovine serum albumin (2 mg/ml), Factor B (0.21 mg/ml) and Factor D (8 μg/ml) in 2 mM-$MgCl_2$ and incubated at 37 °C for 20 min with 100 μl 10% of (v/v) EAC3b (in CFD). The cells (EAC3bBbP) were then pelleted by centrifugation, quickly washed with 1 ml of PBS, and resuspended in 0.5 ml of CFD containing 10 mM-EDTA and bovine serum albumin (3.5 mg/ml). After incubation for a further 80 min at 37 °C, the cells were again centrifuged, the supernatant was removed and the cells were washed once. The radioactivities of the pellet, supernatants and washes were counted to obtain the binding and elution data shown. The data for a parallel sample, incubated in 10 mM-EDTA throughout, are also given. (*b*) Binding of eluted properdin to EAC3b, EAC3bB and EAC3bBb. A 20 μl portion of the EAC3bBb eluate, 20 μl of the unadsorbed fraction and 20 μl of a corresponding fraction of 'activated' properdin were assayed for binding to 2.5×10^7 EAC3b, EAC3bB (42 μg of Factor B/ml) and EAC3bBb (42 μg of Factor B/ml and 1.5 μg of Factor D/ml) in 0.2 ml of CFD containing 0.2 mM-$NiCl_2$, 2 mM-$MgCl_2$ and bovine serum albumin (4 mg/ml). The results shown are averages for duplicate 0.1 ml samples assayed for binding after incubation for 20 min at 37 °C.

(*a*) Binding to, and elution from, EAC3bBb

	Percentage bound	Percentage eluted
EAC3bBb	55.0	53.4
EAC3b + Factor B + Factor D + EDTA	4.1	3.2

(*b*) Binding of eluted properdin to EAC3b, EAC3bB and EAC3bBb

	Percentage binding to: EAC3b	EAC3bB	EAC3bBb
'Native' properdin	2.6	28.9	55.7
EAC3bBb eluate	2.3	28.9	47.7
'Activated' properdin	35.4	63.7	63.1

(Götze & Müller-Eberhard, 1974), such an isolation procedure was monitored by using serum to which a portion of radiolabelled unfractionated properdin had been added. In this experiment 9.2% of the added properdin was fixed to limiting amounts of yeast cell walls (1 h incubation at 15 °C), and 8.3% was recovered after elution with CFD containing 10 mM-EDTA and 0.65 M-NaCl (1 h at 37 °C). The elution profiles on Fractogel TSK HW-55(S) of both the eluted and the unadsorbed material are shown in Fig. 6. As can be seen from Fig. 6(*a*), molecules that are eluted early on Fractogel TSK HW-55(S) are preferentially recovered in the adsorbed and eluted rather than in the unadsorbed protein. However, this shift is no more than that indicated in the theoretical profile derived from the selectivity revealed in Fig. 4(*d*) and does not imply any

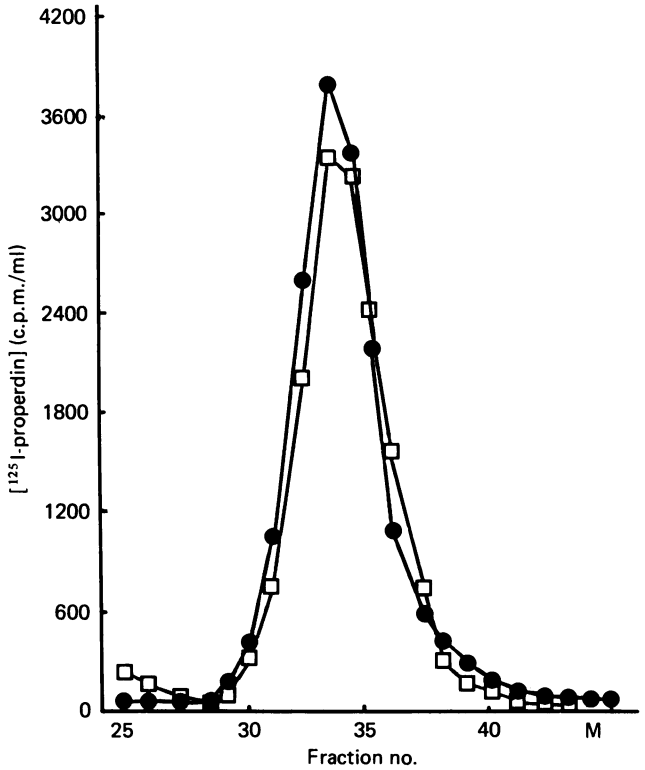

Fig. 5. Non-activation of 'native' properdin by binding to, and elution from, EAC3bBb

A 0.15 ml portion of ^{125}I-labelled properdin eluted from EAC3bBb (□) as described in Table 2 was compared, by chromatography on Fractogel TSK HW-55(S), with 50 μl of the control adsorbed sample of 'native' properdin (●). M indicates the position of the myoglobin peak.

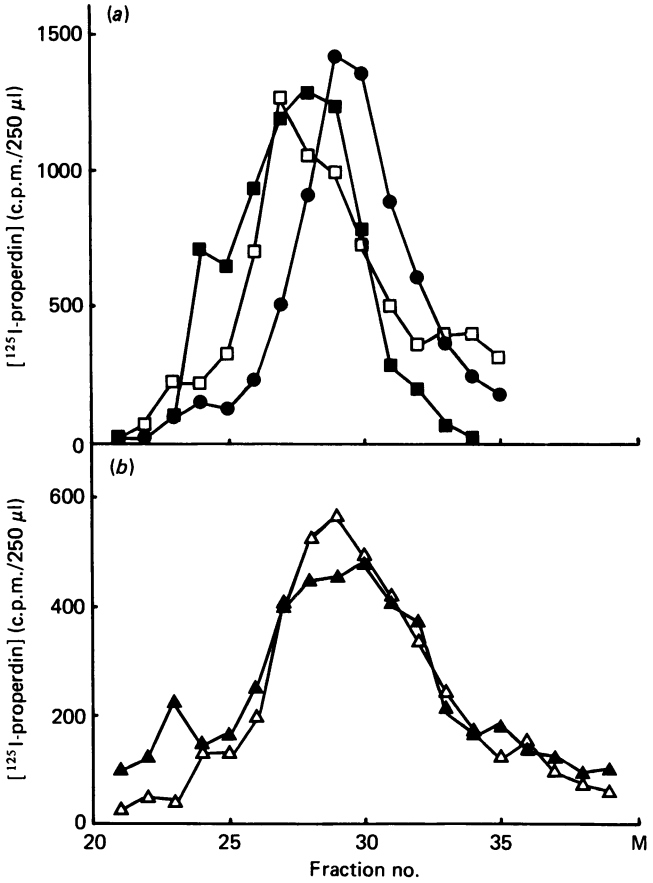

Fig. 6. Re-isolation of ^{125}I-labelled properdin from normal human serum by using adsorption to yeast cell walls

(*a*) Isolation of ^{125}I-labelled properdin from normal human serum by adsorption on yeast cell walls. A 10 ml portion of normal human serum was mixed with 1 μCi of ^{125}I-labelled 'native' properdin. A 0.5 ml portion was retained and analysed by gel filtration on Fractogel TSK HW-55(S) (●), and the remainder was incubated at 15 °C for 1 h with 0.1 ml of 20% (v/v) yeast cell-wall suspension (in CFD). The yeast was removed by centrifugation, washed three times in 10 ml portions of PBS and resuspended in 1 ml of CFD containing 10 mM-EDTA and 0.65 M-NaCl. After incubation for 1 h at 37 °C, the yeast was again removed by centrifugation and 0.25 ml of the eluate was analysed by gel filtration on Fractogel TSK HW-55(S) (□). The theoretical elution profile (■) was derived by multiplying the radioactivity in each fraction of the non-adsorbed-properdin elution by the percentage recoveries of radioactivity given by the corresponding fractions in Fig. 4(*d*). (*b*) Euglobulin precipitation and freeze–thaw of re-isolated properdin. A 0.7 ml portion of the zymosan eluate was dialysed overnight at 4 °C into deionized water, pH 5.5, and the euglobulin precipitate, which contained properdin, recovered by centrifugation at 10000 *g* for 15 min. The pellet was redissovled in 0.5 ml of PBS and then clarified by re-centrifugation. Half of this material was subjected to one freeze–thaw (−78 °C and 37 °C), and 0.2 ml of each half was analysed on Fractogel TSK HW-55(S). △, Euglobulin precipitate; ▲, freeze–thawed euglobulin precipitate.

alteration in properdin consequent on binding to yeast. More importantly, there is no appearance of a peak at the position of 'activated' properdin. The eluted material was then further fractionated by acid euglobulin precipitation (Pillemer *et al.*, 1954), with 98% recovery of radioactivity, and subjected to one cycle of freezing and thawing. Fig. 6(*b*) shows that the appearance of 'activated' properdin followed the freeze–thaw cycle but not euglobulin precipitation.

Electron micrographs of 'native' and 'activated' properdin

A 0.4 mg portion of properdin was fractionated on a pre-blocked Fractogel TSK HW-55(S) column. The absorbance at 215 nm was used to identify properdin peaks, and freshly prepared samples of these were examined by electron microscopy. Fig. 7 shows that the 'native' properdin is composed of ring structures of variable sizes, similar to the structures described by Smith *et al.* (1984). However, these structures are not visible in the 'activated' fractions, where only large amorphous aggregate-like material was found. Both forms could be identified in unfractionated samples.

DISCUSSION

Analysis of the role and mode of action of properdin within the alternative pathway of complement has always been confused by the existence of two forms of the protein, variously described as 'native' or 'precursor' and 'activated'. This problem has been exacerbated by the lack of a procedure for the resolution and, more importantly, recovery of the two. In the present paper we

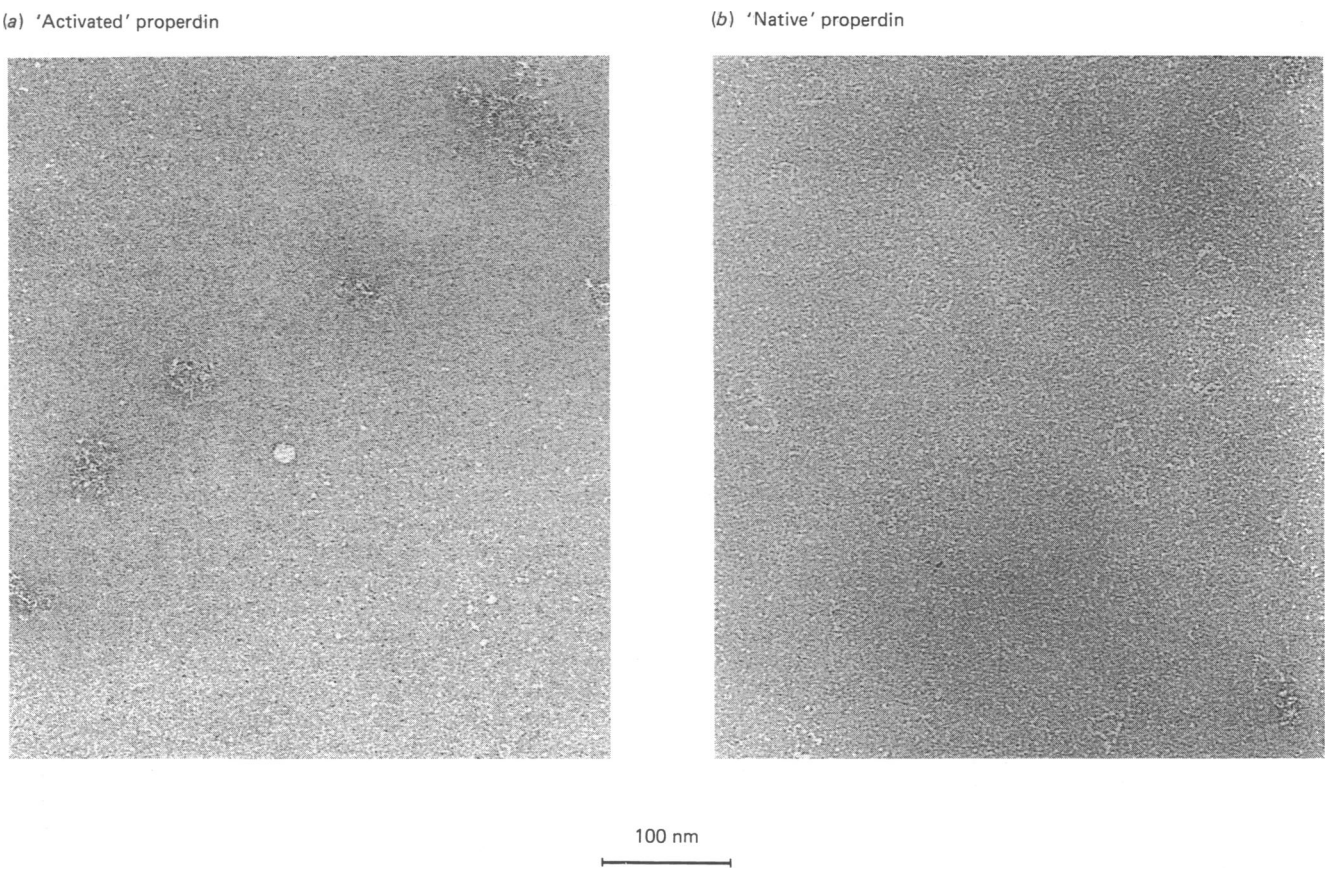

Fig. 7. Electron micrographs of 'native' and 'activated' properdin

A 0.4 ml portion of purified properdin (0.4 mg) was chromatographed on Fractogel TSK HW-55(S), and fractions were screened for absorbance at 215 nm. Droplets of the peak fractions of (*a*) 'activated' and (*b*) 'native' properdin were adsorbed on carbon films. These were washed with 0.15 M-NaCl, negatively stained with 1% (w/v) uranyl acetate and examined by electron microscopy. Micrographs were taken at a magnification of ×61000 and then photographically enlarged to the scale shown.

describe a rapid procedure, using gel filtration on Fractogel TSK HW-55(S), whereby resolution and recovery of both forms may be achieved. This procedure has enabled definition of the functional properties of the two forms and of the relationship between them with a precision that has not formerly been possible.

Fractogel TSK HW-55(S) was selected as, with a fractionation range for globular proteins of 10^4–10^6 Da, it has the potential for resolution of the heterogeneous size variants previously described for properdin. However, in common with other gel-filtration matrices, selective loss of the 'activated' form was seen during chromatography of properdin on this polyvinyl matrix. [The irreversible binding of 'activated' properdin to C3b–Sepharose reported by Medicus *et al.* (1980) is likely to have been largely non-specific binding to the matrix rather than selective binding to C3b.] Loss was characterized by irreversible binding or 'sticking' of protein to the top layer of the column matrix. However, recovery of radiolabelled 'activated' properdin was considerably enhanced (96% compared with 37% recovery) if properdin was loaded on to the column in the presence of gelatin. The mechanism of blocking is not understood. However, some proteins, especially lipoproteins, tend to form a precipitate on gel particles (Fischer, 1969), and it is possible that gelatin blocked sites upon which properdin would otherwise have been adsorbed.

The concept of properdin 'activation' arose because two functionally different forms could be identified, and this concept has persisted in the literature in spite a lack of evidence as to its nature. In addition to adsorption on a C3bBb complex, 'activation' of properdin during isolation has been correlated with acid euglobulin precipitation, cycles of freezing and thawing, and adsorption on ion-exchange matrices (Fearon *et al.*, 1974; Medicus *et al.*, 1976*b*; Götze *et al.*, 1977). The properdin used in the present study had been adsorbed on CM-Sephadex and had been frozen and thawed at least twice during its isolation. When added to freshly drawn serum it also initiated C3 consumption. By accepted dogma it would therefore be regarded as being 'activated' properdin, even though no alternative-pathway-dependent adsorption on a surface had been used in its purification.

Gel filtration of the isolation properdin on Fractogel TSK HW-55(S) produced two peaks, a broad major peak centred at an elution position that corresponds to a molecular mass for a globular protein of about 800 kDa, and a minor peak that was eluted close to the void

volume of the column and therefore has an apparent molecular mass in excess of 10^6 Da. Re-fractionation of portions of each of these peaks showed that they contained stable components, as each maintained its elution position, and that the major peak itself was composed of discrete oligomers. This latter finding is in agreement with the work of others, and its compatible with a heterogeneous mixture of molecules with discrete associations of subunits contained within the main peak (Smith *et al.*, 1984). Although no estimate as to the subunit composition of these oligomers could be made from the gel-filtration data alone [properdin is highly asymmetric, with an anomalous gel-filtration elution position (Pensky *et al.*, 1968; Minta & Lepow, 1974; Smith *et al.*, 1984)], electron micrographs indicated the presence of ring-shaped structures similar to the dimers, trimers and tetramers described by Smith *et al.* (1984). In contrast, the minor high-molecular-mass peak contained only aggregated material, with no visible higher-ordered structure. This is consistent with its larger size as judged by gel filtration. Both amorphous aggregates and defined oligomers were present in the unfractionated material. Analysis by gel filtration of properdin in freshly drawn serum or plasma showed a similar broad peak centred on an apparent molecular mass of 800 kDa, but no minor peak at the void volume.

The above data establish that there exists a stable but heterogeneous association of subunits both in isolated and in unfractionated properdin, and that purified but not unfractionated properdin can also contain a second resolvable high-molecular-mass form of the protein. The properties of the differently sized molecules were therefore analysed and compared with the properties of the 'native' and the 'activated' properdin. The population contained within the major peak bound only weakly to EAC3b or to yeast-C3b at physiological ionic strength, and required the addition of Factor B or both Factors B and D for stable association with C3b-coated surfaces. It did not initiate C3 consumption in serum. In contrast, the protein contained in the minor peak initiated C3 consumption in serum and bound both to yeast-C3b and to EAC3b. Thus it is clear that only the protein of the minor peak has the properties of 'activated' properdin, and that 'activated' properdin can be recognized by virtue of its elution position from Fractogel TSK HW-55(S). Furthermore, 'activated' properdin does not occur at all in unfractionated or unactivated plasma and serum. Although these data suggest 'activated' properdin to be a denatured aggregated form of the protein, it cannot readily be removed by centrifugation (Chapitis & Lepow, 1976; Smith *et al.*, 1984), and can still stabilize C3bBb complexes, thus retaining the major function of the protein.

The identification of the minor early peak as 'activated' and the major peak as 'native' properdin was confirmed by interconversion of the two forms *in vitro*. Medicus *et al.* (1980) showed that mild treatment with denaturants converted 'activated' properdin into 'native' properdin. We have demonstrated that this conversion correlates with a decrease in the amount of the early peak. Similarly, a number of workers have shown that cycles of freezing and thawing increase the proportion of 'activated' properdin in a preparation (Fearon *et al.*, 1974; Medicus *et al.*, 1980). Again, this correlates with an increase in the amount of protein contained in the early peak. It is therefore probable that isolation of 'native' rather than 'activated' properdin has largely been consequent on improved protein isolation technology, and that most preparations described in the literature as being 'activated' on the basis of initiating C3 consumption in serum or of some binding to C3b were predominantly 'native' and contained only a minority of 'activated' molecules. For example, Smith *et al.* (1984) described their preparation of properdin as partially activated, yet electron micrography revealed only the cyclic structures found by us in the major peak of 'native' properdin.

The possibility that 'activated' properdin might reflect a true physiological state of the protein trapped while the bulk of properdin reversed to a precursor state after release from a surface-bound C3bBb enzyme was also investigated by using gel filtration on Fractogel TSK HW-55(S). However, when 'native' radiolabelled properdin was added either to serum and then yeast added or to EAC3bBb, the cells or particles were washed, and then properdin was released during decay of the convertase in EDTA, essentially all of the bound properdin was recovered and had an unaltered, 'native', gel-filtration profile. In addition, analysis of the released material for the properties of 'activated' properdin confirmed that no activation had taken place. Further attempts to generate 'activated' properdin from protein released from serum-treated yeast using acid euglobulin preciptation were also unsuccessful, and it was not until a cycle of freezing and thawing had been included that 'activated' protein could be detected. We have therefore been unable to generate physiologically 'activated' properdin.

The studies of properdin binding to and recovery from C3b-, C3bB- and C3bBb-coated surfaces reported here have, however, indicated an explanation for an apparent alteration in the properties of properdin eluted from alternative-pathway-activating surfaces. Although 'native' properdin binds only weakly to C3b at physiological ionic strength, this binding is increased at low ionic strength. Under these conditions we have demonstrated a difference in avidity of higher and lower oligomers for C3b-, C3bB- and C3bBb-coated erythrocytes, with the early-eluted species (presumably higher-order oligomers) having the greater avidity. [This gradation in avidity for C3b could account for the similar findings reported by Smith *et al.* (1984), although it is likely that some 'activated' material was also contained in their early fractions from Sephacryl S300.] We have shown that high-avidity molecules would be selectively adsorbed from serum on limiting amounts of yeast, and it is likely that in any preparation involving adsorption on and elution from an alternative-pathway-activating surface that the increased avidity of higher oligomers for the surface will result in their relative concentration in the recovered material. The degree of concentration will depend on the amount of available bound C3bBb and the nature of washing procedures, and could well result in an apparent change in the properties of the isolated properdin. However this material would not be 'activated', as it would not agglutinate EAC3b, nor would it initiate C3 consumption in serum. (Additional selection during isolation might occur with loss of 'activated' forms on column matrices, and by selective pooling of column fractions.)

These findings permit a simplified and more rational view of the role of properdin. As the 'native' form is

sufficient to stabilize the alternative-pathway C3 convertase, and can be quantitatively recovered from the convertase, any 'activation' step is unnecessary. Indeed, 'activated' properdin, if formed and released, would lead to unregulated C3 consumption in serum unless a mechanism for its 'deactivation' existed. Although we cannot rule out the possibility that there is a conformational transition in C3bBb-bound properdin, this would have to be fully reversible. There is circumstantial evidence that such a transition would not in any case have any relationship to the 'native' and 'activated' forms analysed here, as Wilson *et al.* (1984) have demonstrated differential sensitivities of 'native'-and 'activated'-properdin-stabilized C3 convertases to sulphated glycosaminoglycans. Our data demonstrate that properdin 'activation' is an artifact of isolation. Although it can be reproduced by freezing and thawing, it does not occur on complement activation. Formation of 'activated' properdin probably reflects aberrant association of properdin monomers or disrupted oligomers after disruption of otherwise stable ring structures by mechanical stress. These larger structures would contain a high number of potential C3b-binding sites, possibly in anomalous spatial relationships, which could give rise to agglutination of EAC3b-bearing cells and, by providing a focus for assembly of stabilized C3 convertases in the fluid phase, initiate C3 consumption. Only the 'native' form exists *in vivo*, and consists of cyclic oligomers (mainly dimers, trimers and tetramers). Although the gradation of avidity within these molecules will give rise to populations of the isolated protein with varied properties, the physiological properties of properdin are those of 'native' properdin, and are confined to the recruitment on to and stabilization of surface-bound C3bB and C3bBb complexes.

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