

THE ROLE OF PROPERDIN IN THE ALTERNATE PATHWAY OF COMPLEMENT ACTIVATION*†

BY OTTO GÖTZE§ AND HANS J. MÜLLER-EBERHARD

(From the Department of Experimental Pathology, Scripps Clinic and Research
Foundation, La Jolla, California 92037)

(Received for publication 7 September 1973)

In earlier work on the properdin system only properdin proper was obtained in highly purified form (1), but its role and position in the reaction sequence which leads to activation of C3-C9 was not elucidated. Because of the similarity of the C3 activator system (2) and the properdin system, we investigated the possible role of properdin in the activation of C3 and C5 via the alternate pathway. In this communication we wish to report that properdin acts as an essential factor in the early reaction steps of the C3 activator system. It appears to function as a modulator which enables native C3 to activate C3 proactivator convertase (C3PAse).¹

We have previously shown that C3PAse causes conversion of C3 proactivator (C3PA) to its enzymatically active form, the C3 activator (C3A), and that this enzyme cleaves C3 into C3a and C3b (2). We also reported that C3PAse acquires enzymatic activity through interaction with C3b and that therefore part of the alternate pathway is governed by a positive feedback mechanism (3), a concept recently emphasized by Nicol and Lachmann (4). Consequently, the question arose as to the manner in which C3PAse is switched-on by activators of the alternate pathway before the positive feedback becomes operative through production of C3b. Is properdin involved in the initial activation of C3PAse?

Many similarities between the C3 activator system and the properdin system (5) have become apparent. Isolated C3PA could be equated with Factor B of the properdin system (6, 7). C3, the hydrazine-sensitive factor (HSF) of the C3 activator system (3), was found to be the HSF of the properdin system or Factor A (8, 9). Also, both systems were shown to participate in the killing of nonsensitized gram-negative bacteria (1, 2) and in the lysis of erythrocytes from patients with paroxysmal nocturnal hemoglobinuria (10, 11). Thus, the earlier findings and those reported in this paper

* This is publication no. 754 from the Department of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, Calif. 92037.

† This work was supported by United States Public Health Service Grant AI-07007 and United States Atomic Energy Commission Contract AT(04-3)-730.

§ Dr. Götte is an Established Investigator of the American Heart Association.

¹ *Abbreviations used in this paper, with synonyms used by other investigators:* C3PA, C3 proactivator (GBG, Factor B); C3A, C3 activator (GGG, β_2 -glycoprotein II); C3PAse, C3PA convertase (GBGase, Factor D); HSF or C3, hydrazine-sensitive factor or the third component of complement (Factor A); HSFa or C3b, activated HSF or the b-fragment of C3; P, properdin; \bar{P} , activated properdin; C3b INA, C3b inactivator (KAF); CVF, cobra venom factor; WHS, whole human serum; and HSA, human serum albumin.

appear to establish the identity of the C3 activator system with the properdin system. The results have been published in the form of an abstract (12).

Materials and Methods

Polysaccharides.—Inulin CP, purchased from Pfanstiehl Laboratories Inc. (Waukegan, Ill.), was suspended to 50 mg/ml in sterile saline. Zymosan, obtained from Nutritional Biochemicals Corp. (Cleveland, Ohio), was prepared according to the method described in reference 13 and was made up to 50 mg/ml saline.

Purified Proteins and Complement Reagents.—Human serum albumin (HSA) was purchased from Behringwerke AG (Marburg, Germany) and cytochrome C from Calbiochem (San Diego, Calif.). Clq (14), C3 (15), and cobra venom factor (CVF) (16) were purified according to published methods. C3b, the activated form of the hydrazine-sensitive factor (HSFa), was prepared by either of two published methods (3, 17). C3PAse was purified from the pseudoglobulin fraction of human ACD-plasma by a series of column chromatographic steps using successively Bio Rex 70 (Bio-Rad Laboratories, Richmond, Calif.), carboxymethylcellulose (Whatman CM-32, Reeve Angel, Clifton, N.J.), and Sephadex G75 (Pharmacia Fine Chemicals, Piscataway, N.J.). The final product was proved to be homogeneous by alkaline gel electrophoresis in 6% polyacrylamide. Details of the purification method will be published separately. When necessary, purified components were reconstituted with respect to divalent cations by the addition of 0.1 vol of a solution of 0.01 M CaCl_2 –0.02 M MgCl_2 in saline.

Human serum (Shis.) genetically deficient in C2 was kindly provided by Dr. Vincent Agnello. C3PA-depleted serum was prepared by heating fresh human serum to 50°C for 30 min. Human serum deficient in C3 was prepared by treatment with hydrazine (3). C3PAse-depleted serum was obtained by passage of 20 ml of fresh serum over a 4.5 × 110 cm Sephadex G75 column equilibrated in 0.15 M NaCl, 0.002 M EDTA, pH 7.3. The bulk of the proteins eluting in two peaks was pooled and concentrated to the original serum volume. C3PAse elutes after the second protein peak and is thereby separated from all known components of complement and the C3 activator system. Human serum specifically depleted of properdin (P) was obtained using an immune adsorbent prepared from the IgG fraction of a monospecific rabbit antiserum. This was done as follows: The globulins of 37 ml of antiserum to human P were precipitated at 40% saturation of $(\text{NH}_4)_2\text{SO}_4$. The precipitated proteins were redissolved in and dialyzed against 0.005 M PO_4 , 0.002 M EDTA, pH 7.3, containing NaCl to give a conductivity of 4 mmho/cm. A 2.5 × 40 cm column of DE32 cellulose, equilibrated with the same buffer, was charged with the dialyzed proteins and eluted with the 4 mmho buffer. The proteins not retained by the column were collected and concentrated to 15 ml representing a total of 78 mg of protein. After dialysis against 0.2 M acetate buffer (pH 5.0), 700 mg of HSA was added and both proteins were copolymerized using 0.5 ml of ethyl chloroformate (J. T. Baker Chemical Co., Phillipsburg, N.J.) according to the procedure of Avrameas and Ternynck (18). After the last wash with phosphate-buffered saline containing 0.002 M EDTA, the immune adsorbent particles were resuspended in fresh human serum to which 0.1 vol of 0.2 M EDTA, pH 7.2, had been added. The mixture was slowly stirred at 4°C for 1 h. The supernatant serum recovered by high speed centrifugation was frozen immediately at –70°C in small aliquots. 50 ml of human serum could be depleted of P by this procedure as evidenced by double diffusion in 1% agarose gel by using a monospecific antiserum to P. Hemolytic titration of the serum before and after adsorption showed a 15% loss of CH_{50} /ml.

Antisera.—Monospecific antisera to Clq, C3, C3PA, and P were raised in rabbits. The first lot of anti-P serum was a gift from Dr. John T. Boyer. It was used to establish the isolation procedure of P. Later an antiserum to P was prepared in this laboratory and was used in all other experiments. Both antisera showed the same specificity for P in human serum as

well as in its isolated form. Rabbit antisera to human IgM, IgG, and IgA, all H-chain specific, were purchased from Behringwerke AG.

Analytical Methods.—Immuno-electrophoresis was performed on microscope slides in 1% agarose (Seakem, Marine Colloids, Inc., Springfield, N.J.) or 1% ion agar no. 2S (Colab, Glenwood, Ill.) in veronal buffer, $T/2 = 0.05$, pH 8.6, containing 0.01 M EDTA. Immunological double diffusion was done in 1% agarose in veronal buffered saline, pH 7.4, containing 0.02 M EDTA. Polyacrylamide electrophoresis was performed in 6% gels in 2-amino-2-methyl-1,3-propanediol (AMPD) buffer as described by Tamura and Ui (19). Gels were prepared in AMPD-HCl buffer, pH 9.5, and the electrode buffer was AMPD-glycine, pH 8.8. For sucrose density gradient ultracentrifugation, the method of Kunkel (20) was followed. Gradients were prepared with a Buchler gradient maker, and after application of the samples the tubes were centrifuged in a Spinco SW 50 rotor for 16 h at 39,000 rpm (Spinco Division, Beckman Instruments, Inc., Palo Alto, Calif.). Protein was determined using the Folin method (21).

Hemolysis.—Sensitized sheep erythrocytes carrying $C4^{oxY}2$ or $C4^{oxY}23$ were prepared and used for effective molecule titrations of C3 and C5 as described previously (22, 23). Total hemolytic complement was determined by a standard method (24).

RESULTS

Purification of Properdin from Human Serum without the Use of Zymosan.—Properdin was purified from the euglobulins precipitated from fresh ACD-plasma or serum by dialysis against 0.008 M EDTA, pH 5.4. The redissolved euglobulins were then successively fractionated on a 2.5×40 cm column of CM-Sephadex C50, a 2.5×40 cm column of CM32 cellulose, and a 5×110 cm column of Sepharose 6B (Pharmacia Fine Chemicals). Column effluents were assayed immunochemically for P. The purification steps are summarized in Table I. Sometimes an additional passage over a 3×115 cm column of Sepharose 6B was necessary to obtain a product of high purity. Large amounts of serum (5–7 liter) are required for a single preparation of purified P because of the low yields which are only 0.5–1% assuming a P concentration in serum of 25 μ g P/ml (25). Therefore, the euglobulins which are not retained by DE32

TABLE I
Isolation of Properdin (P) from Human Serum

Precipitation of the euglobulins:	Dialysis of fresh serum against 0.008 M EDTA, pH 5.4.
Chromatography on CM-Sephadex C50:	0.02 M PO_4 , 0.002 M EDTA, pH 6.0. NaCl gradient to 50 mmho. Elution of P: 22–27 mmho.
Chromatography on CM-cellulose: (CM32)	0.02 M PO_4 , 0.002 M EDTA, pH 6.0. NaCl gradient to 30 mmho. Elution of P: 11–15 mmho.
Chromatography on Sepharose 6B:	0.5 M NaCl in 0.05 M Tris-HCl, 0.002 M EDTA, pH 8.0.

cellulose at 0.03 M PO_4 , pH 8.1, NaCl to 4.2 mmho/cm, a step which is used in the laboratory for the isolation of C3 and C5, are often combined and used as the starting material for P preparations. The last step of the isolation procedure, chromatography on Sepharose 6B, often yields two P-containing peaks (Fig. 1). Since by immunochemical and activity criteria the properdin in the two peaks appeared to be identical, it is assumed that the earlier eluting peak contains aggregates of P the size of which was not determined. The homogeneity of the final product was demonstrated by polyacrylamide electrophoresis (Fig. 2) and by immunoelectrophoresis (Fig. 3). Purified P at a concentra-

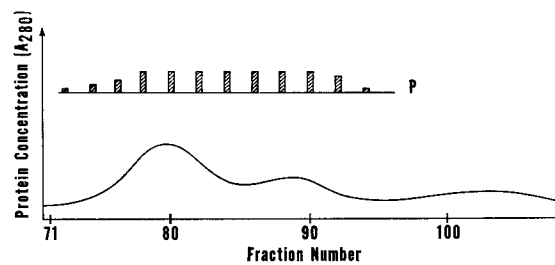


FIG. 1. The final step of the isolation of properdin: elution of P from Sepharose 6B. The column was continuously monitored at 280 nm for protein. Fractions of 5 ml each were collected and every second fraction was immunochemically tested for P (bars).

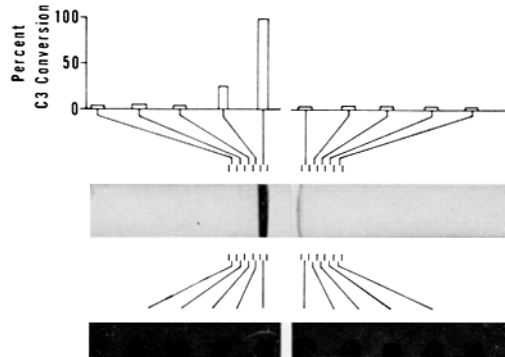


FIG. 2. Analysis of isolated P by polyacrylamide gel electrophoresis, using two gel columns, one for protein staining and the other for elution of biologically and antigenically active material. 160 μg of P were dialyzed against AMPD buffer, pH 9.5, mixed with sucrose (10% final concentration) and 80 μg each were layered on top of the 6% running gels. Another 6% running gel was then polymerized on top of each liquid sample. After electrophoresis (the anode was at the right) the two segments of one gel were stained, whereas the two segments of the second gel were sliced into 2-mm sections as indicated. The sections were eluted overnight at 4°C with 0.1 ml of phosphate-buffered 0.3 M NaCl, pH 7.4, containing 0.1% gelatin. The eluates were then assayed for C3 converting activity by incubation with fresh serum followed by immunoelectrophoretic analysis of C3 (top). P was detected in the same eluates by double diffusion in agarose using a monospecific antiserum (bottom).

tion of 0.5 mg/ml did not react with rabbit antisera to human Clq, IgG, IgA, or IgM as tested by double diffusion in agarose. Upon sucrose density gradient ultracentrifugation, P from the second peak of the Sepharose 6B column (Fig. 1) was found to sediment with an s -rate of 5.4S (Fig. 4).

The Activity of Isolated Properdin.—When fresh normal human serum or human serum genetically deficient in C2 were incubated with isolated P, the conversion of C3PA and of C3 could readily be demonstrated by immunoelectrophoresis. In contrast, C3 and C3PA conversion was observed neither when P was incubated with isolated C3 and/or isolated C3PA, nor when P was added to serum rendered deficient in C3, C3PAse, or C3PA. The deficient sera were fully restored with respect to the effect of P upon addition of the respec-

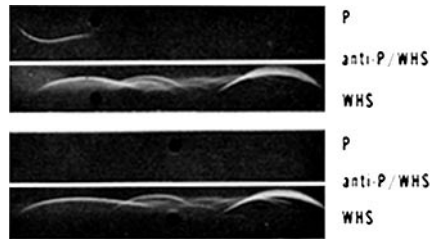


FIG. 3. Immunoelectrophoretic representation of isolated properdin. Purified P at a concentration of 0.5 mg/ml was analyzed in 1% agarose (upper panel) or 1% ion agar (lower panel). For comparison, fresh human serum was included on the slides which were developed with a mixture of rabbit anti-P/anti-whole human serum. As can be seen, agar proved to be unsuitable for the detection of P under the conditions used. The anode was at the right.

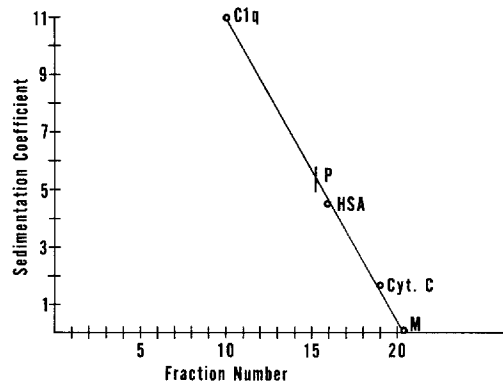


FIG. 4. Analysis of purified P by sucrose density gradient ultracentrifugation. The value for P (5.4S) is an average derived from three separate experiments (5.2S, 5.5S, and 5.6S). The position of P in the gradient fractions was determined by its capacity to convert C3 in WHS as well as by immunochemical analysis. Both assays located P in the same gradient fractions. Markers used were Clq and HSA (11S and 4.5S, both detected immunochemically), cytochrome C (1.7S, detected by its adsorbance at 412 nm), and the meniscus (M).

tive isolated proteins. This is exemplified for C3PA-deficient serum in Fig. 5. When mixtures containing highly purified P, C3, C3PA, and isolated C3PAse were incubated together in the presence of 10^{-3} M magnesium ions, complete conversion of C3 and nearly complete conversion of C3PA was consistently demonstrable. Omission of any one of the purified proteins or the addition of EDTA prevented both conversion of C3 and of C3PA (Fig. 6).

Impairment of the C3 Activator System by Specific Depletion of Serum Properdin.—Fresh serum was depleted of P by immune adsorption and reconstituted with respect to divalent cations. When inulin, one of the known activators of the system, was incubated with P-depleted serum, conversion of C3 could not be demonstrated, indicating that P is essential for the activation of C3 by the alternate pathway. This finding is consistent with the results of the above described experiments in which isolated components had been used. In contrast,

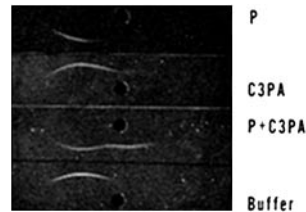


FIG. 5. Dependence of C3 conversion by P on the presence of C3PA. C3PA-depleted (heated) serum was incubated for 30 min at 37°C with isolated properdin ($10\ \mu\text{g}/\text{ml}$ serum) in the absence and in the presence of isolated C3PA ($100\ \mu\text{g}/\text{ml}$ serum). Two controls, one receiving only C3PA, the other one only buffer were included. The mixtures were then assayed for C3 conversion by immunoelectrophoresis in 1% ion agar using an antiserum monospecific for C3. The anode was at the right.

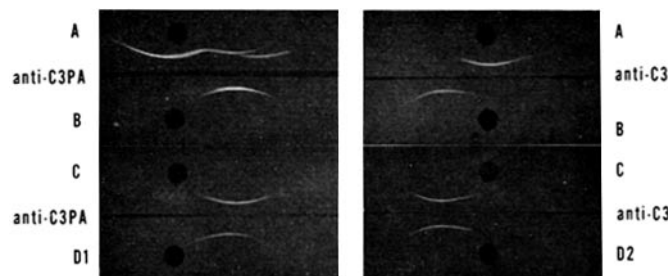


FIG. 6. Immunoelectrophoretic demonstration of P-induced conversion of isolated C3PA and isolated C3. Reaction mixtures ($30\ \mu\text{l}$) contained: (A) $0.4\ \mu\text{g}$ P, $13\ \mu\text{g}$ C3, $6\ \mu\text{g}$ C3PA, and $0.08\ \mu\text{g}$ C3PAse; (B) C3, C3PA, and C3PAse, but no P; (C) P, C3, C3PA, but no C3PAse; (D1) P, C3PA, and C3PAse, but no C3; (D2) P, C3, and C3PAse, but no C3PA. Incubation was 30 min at 37°C . Gels were loaded with $20\ \mu\text{l}$ of the reaction mixtures which were analyzed for conversion of C3PA in 1% agarose (left) and for conversion of C3 in 1% ion agar (right). A new anti-C3PA antiserum which detects both fragments of C3PA was used. The anodes were at the right.

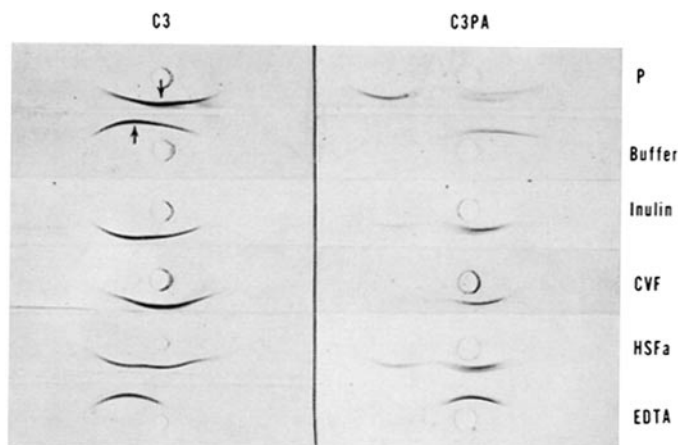


FIG. 7. Conversion of C3 and C3PA in P-depleted serum by various activators. 0.050 ml of P-depleted serum was incubated for 30 min at 37°C with 1 μ g of isolated P, 0.25 mg of inulin, 5 μ g CVF, or 2 μ g HSFa (C3b). Controls received buffer or EDTA (0.002 M final concentration). Total reaction volumes were 0.065 ml. After incubation the mixtures were analyzed for C3 conversion (left) and for C3PA conversion (right). Slides were stained with amido black. The anodes were at the right.

treatment of P-depleted serum with isolated P, CVF, or C3b (HSFa) resulted in C3 and C3PA conversion (Fig. 7). Heat-aggregated human IgG, an activator of the classical pathway, was fully able to trigger C3 conversion in the absence of P.

Consumption of C3 by P in P-Depleted and C2-Deficient Serum.—In order to establish the quantitative relationship between the degree of activation of C3 and the amount of P required, experiments were performed in which graded amounts of isolated P were incubated with P-depleted serum for 30 min at 37°C. The consumption of hemolytically active C3 by each amount of P was then determined with EAC4^{oxy}2 cells. A linear relationship was observed between C3H₅₀ consumption and the input of P when plotted in terms of $y/(1 - y)$ vs. μ g properdin. When the data were expressed in percent consumption of effective C3 molecules, as shown in Fig. 8, C3 consumption increased with increasing amounts of P. The highest amount of P used (1.5 μ g/0.05 ml of P-depleted serum) is similar to that reported to occur in whole human serum (25). Several different activators of the alternate pathway were compared with regard to their effects in C2-deficient and P-depleted serum. The results are given in Table II.

Consumption of C5 by P in P-Depleted Serum.—Early during our investigation it had been observed that P, although efficient in activating C3, initiated consumption of C5 in normal human serum only to a very small extent (approximately 10%). Experiments dealing with the activation of C5 were therefore repeated and extended using P-depleted serum. It was found that there

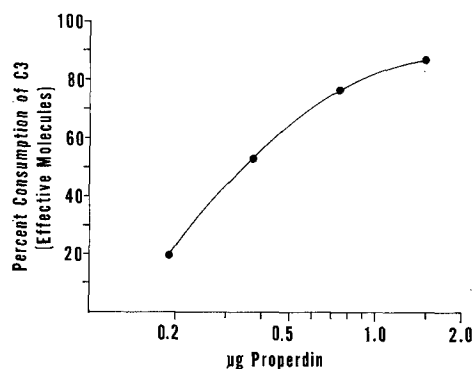


FIG. 8. Quantitative relation between C3 consumption in P-depleted serum and amount of added purified P. Increasing amounts of purified P were added to 0.050 ml of P-depleted serum and were incubated for 30 min at 37°C in a total reaction volume of 0.080 ml. C3 consumption was then determined by effective molecule titration.

TABLE II
Consumption of C3 in Properdin (P)-Depleted and in C2-Deficient Serum

Serum	Activator	Concentration	Consumption of C3*
		mg/ml	%
Properdin-depleted	P	0.08	94.9
	CVF	0.16	94.0
	Inulin	5.00	10.1
	P plus	0.08	100.0
	Inulin	5.00	
	C3b	0.40	43.0
C2-deficient	P	0.06	43.7
	P plus	0.06	64.5
	Inulin	5.00	
	Inulin	5.00	27.0

* Measured as effective molecules.

was little C5 consumption except with the highest amounts of P used (about 15% consumption with 1.5 µg P/0.05 ml serum). If, however, the reaction was performed in the presence of 0.25 mg zymosan, efficient consumption of C5 was demonstrable, reaching about 40% at less than 0.04 µg P/0.05 ml serum. Incubation of larger amounts of P in the presence of zymosan resulted in a marked inhibition of this consumption (Fig. 9).

DISCUSSION

The present studies were initiated in order to evaluate the possible role of properdin in the C3 activator system and thereby to clarify the relationship

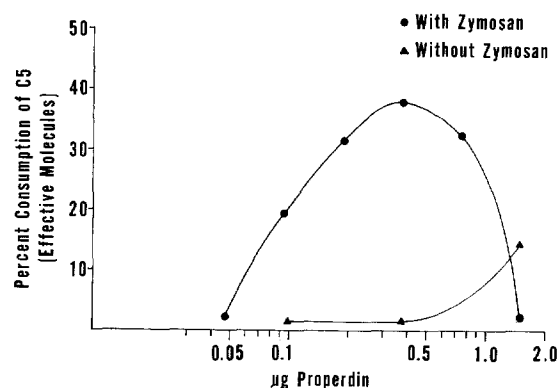


FIG. 9. Quantitative relation between C5 consumption in P-depleted serum and amount of added purified P in the presence and absence of zymosan. Increasing amounts of purified P were added to P-depleted serum in the absence or the presence of 0.25 mg of zymosan and were incubated for 30 min at 37°C in a total reaction volume of 0.080 ml. C5 consumption was then determined by effective molecule titration. The values obtained by incubation with P and zymosan were corrected for the amount of C5 consumed by incubation with zymosan alone which was between 8–12%.

of this system to the properdin system. Because it was thought probable that P purified by adsorption to zymosan according to Pensky et al. (1) might be in an altered state, a purification procedure based on conventional chromatographic methods was designed. Using this procedure highly purified properdin was obtained which showed molecular homogeneity upon physicochemical and immunochemical analysis. Properdin is a highly basic plasma protein which exhibits a net positive charge during polyacrylamide electrophoresis at pH 9.5. Its *s*-rate upon sucrose density gradient ultracentrifugation was found to be 5.4S. These results are in agreement with those published in 1968 for zymosan purified P by Pensky et al. (1) who found a sedimentation coefficient of 5.2–6.4S and a molecular weight of 223,000 daltons. Recently, Minta and Lepow (26) reported a molecular weight of 184,000 daltons for zymosan-purified properdin. The protein was found to be composed of four noncovalently linked subunits of 45,000 daltons each. In our experiments, P eluted from gel permeation chromatography columns with an elution volume between that of Clq and IgG. All those data are suggestive of a molecule with a high frictional ratio. Properdin apparently has a propensity to form aggregates (Fig. 1), a behavior which might explain the higher sedimentation coefficients (from 10S to 27S) which have been reported in the earlier literature (5, 27–29).

All P preparations obtained by the described method caused immunoelectrophoretic conversion of C3PA and of C3 as well as consumption of hemolytically active C3 when incubated with fresh serum or P-depleted serum in the absence of activating substances such as inulin. In order to evaluate the precise requirements for these reactions, several experiments using isolated, highly

purified components were performed. In view of the known role of C3b as an activator of C3PAse, special care was exercised to utilize only those protein preparations which were entirely devoid of C3b. It was found that C3PA conversion occurred in the isolated system only when properdin, C3, C3PAse, and magnesium ions were present. Similarly, C3 conversion required P, C3PAse, C3PA, and magnesium ions (Fig. 6). Inulin could not replace P in these reactions.

It is conceivable that the observed effects of P in fresh serum as well as in the isolated system are, at least in part, due to contamination of the isolated protein with endotoxins which are known to be potent activators of the C3 activator system. The results of sucrose density gradient ultracentrifugation seem to argue against this possibility, since both the P antigen as well as the P activity were found to sediment congruently with 5.4S, whereas contaminating endotoxins would be expected to sediment at a faster rate.

An absolute requirement for P in the alternate pathway could be demonstrated with the use of specifically P-depleted serum. Whereas activators which interact directly with C3PA or C3PAse, such as CVF and C3b, were still able to initiate C3 conversion, inulin was virtually unable to trigger the system in the absence of P (Fig. 7).

These results show that two groups of activating substances of the alternate pathway may be distinguished. One group of substances is capable of acting independent of P (CVF and C3b). Other activators such as inulin require participation of P. This applies probably also to other polysaccharides, endotoxins, IgA aggregates (30, 31), and the nephritic factor (32). Results pertaining to some of these aspects were also presented by others (33, 34). The results further show that the isolated properdin was obtained in an activated state from a precursor and that it differs functionally from the form in which it exists in plasma or fresh serum. Precursor properdin can become activated after addition of substances such as inulin, zymosan, endotoxins, or IgA aggregates to serum, either by direct interaction or by mediation of additional serum factors. The latter possibility has been raised by Spitzer and Stitzel (35). Since there is as yet no evidence that activated P (\bar{P}) can cleave C3 directly to generate C3b which is known to activate C3PAse, it is postulated that P interacts with native C3 in a way which causes expression of C3PAse activity. This enzyme would then activate C3PA which in turn cleaves C3, thereby initiating the C3b-dependent positive feedback mechanism of the C3 activator system (3). Our concept of these reactions is schematically depicted in Fig. 10.

Purified P efficiently triggers the activation or consumption of C3 in P-depleted or fresh serum in the absence of particulate polysaccharides. C5 activation by \bar{P} , however, is not efficiently initiated in the absence of inulin or zymosan and is markedly inhibited at higher \bar{P} concentrations when zymosan is present (Fig. 9). The effect of insoluble polysaccharide on the formation of a P-dependent C5 convertase might be due to a stabilization of molecular interac-

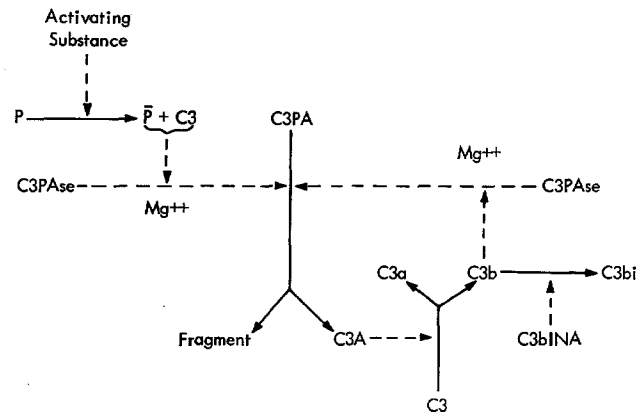


FIG. 10. Present concept of the molecular mechanisms of the alternate pathway of complement activation.

tions between several proteins through binding to a surface. Inhibition of the C5 convertase by larger amounts of P might be explained on the basis of steric hindrance at the site of assembly of this multicomponent enzyme.

The present study assigns to P an essential role during the early reactions leading to the activation of C3–C9 via the alternate pathway. It does not, however, exclude the possibility that P is also required for the formation of the C5 convertase and that it might be an integral part of this complex enzyme.

SUMMARY

Properdin (P), a highly basic euglobulin, was purified from human serum to molecular homogeneity without the use of zymosan. Isolated P was found to efficiently initiate activation of the alternate pathway of complement activation (C3 activator or properdin system) and to be an essential component during its early reaction stages. The activity of isolated P did not require the presence of an activating polysaccharide. It was therefore concluded that purified P had been obtained in an activated form (\bar{P}).

In an isolated reaction system containing purified C3, C3 proactivator (C3PA), and C3 proactivator convertase (C3PAse), \bar{P} was able to mediate the activation of C3PAse which in turn activated C3PA to cleave C3. This activation of C3PAse was found to depend on the presence of native C3. These results allowed the formulation of a concept in which \bar{P} is envisaged to act as a modulator of native C3 enabling it to activate C3PAse.

Activation of C3 was efficiently mediated by \bar{P} in the fluid phase. Efficient activation of C5, however, required the participation of an insoluble polysaccharide (zymosan). The possibility is raised therefore that \bar{P} might also be an integral part of the multimolecular C5 convertase of the alternate pathway of complement activation.

We are indebted to Mrs. Margaret J. Schroeder and Mrs. Cynthia Stone for skillful technical assistance.

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