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Isolation of Properdin From Human Plasma.*† (23453)

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(Introduced by J. R. Pappenheimer)
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The presence in human serum of a protein, properdin, which together with complement or substances resembling complement, in the presence of Mg^{++} constitutes a natural defense mechanism of blood, was first reported by Pillemer and coworkers(1). A procedure was described for the separation and concentration of properdin activity from human serum. The procedure involves adsorption of properdin on the insoluble yeast polysaccharide, zymosan, in the presence of other serum factors and Mg^{++}(2).‡

The magnesium ion concentration essential for adsorption of properdin is absent from collected human plasma, either because magnesium is bound by a chelating agent such as sodium citrate or because the cations have been removed by adsorption on a cationic exchange resin. For this reason, Pillemer and his colleagues studied the isolation of properdin only from human serum where the cationic constituents of blood are essentially unchanged. To make properdin available in the quantities essential for its careful clinical and chemical characterization, a scheme for its preparation from plasma without alteration of other plasma components is desirable. Whereas schemes of plasma fractionation permitting the isolation of many components from a single plasma pool have been in common use for the preparation of plasma fractions on a

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‡ Pillemer, L., and Blum, L., to be published.

large scale since World War II, serum has not been similarly fractionated since all the components of the clotting system have been removed or altered in its preparation. Plasma is thus available in quantity in many laboratories while serum is not.

The present paper reports the successful application of the procedure described for serum to plasma from blood collected through a cationic exchange resin and to plasma from blood collected in ACD solution. Certain modifications have been introduced which increase the efficiency of the technic in terms of time and volume. Preliminary studies which indicate the successful separation of properdin activity from plasma without the use of zymosan are reported.

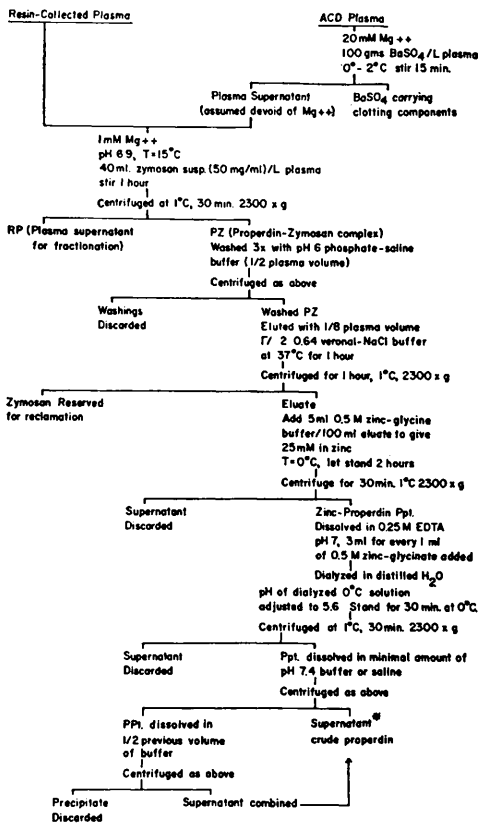
Materials. The blood employed was collected either in ACD solution, (acid-citrate-dextrose solution, prepared according to NIH formula A) or through cationic exchange resins (IRC-50 or Dowex-50) in the ADL-Cohn Blood Fractionator(3). Zymosan was obtained commercially.§ Water baths capable of maintaining the desired temperature to ± 1°C were used for temperature control during both adsorption and elution of properdin. Zinc glycinate(4) reagent was used for the introduction of zinc ions. The stock barbital buffer for suspension of zymosan has been used as described(5), with the exception that magnesium and calcium are omitted. The barbital buffer used during elution is as described(5). *Procedures.* The zymosan adsorption technic for the preparation of properdin described by Pillemer *et al.*(5) was followed closely with only those modifications specifically noted. The procedure has been found to be perfectly reproducible. Application of the procedure to human plasma involves the maintenance of a sufficient concentration of Mg^{++} ions without the induction of clotting. *A. Application to Resin-Collected Plasma.* Under the standard conditions of flow rate and contact time used in collecting blood by passage over a sodium cycle cationic exchange resin, the concentration of Ca^{++} + Mg^{++} ions is reduced to approximately 5 x 10^{-5} M(6). Addition of Mg^{++} ions to resin-collected plasma to a concentration of 10^{-3} M does not initiate any overt coagulation process. The zymosan adsorption of properdin may then follow with successful removal of properdin activity from the plasma. In 20 runs, in which the resin-collected plasma had an average properdin activity of 4 units per ml,‖ less than 1 unit per ml of properdin remained in the plasma after zymosan adsorption. *B. Application to ACD Plasma.* In the presence of sodium citrate, magnesium and other cations are bound by the citrate ions. Saturation of the citrate ions with added Mg^{++} ions leads to liberation of Ca^{++} ions, the association constant of the Ca-citrate complex being smaller than that of the Mg-citrate complex. The liberated Ca^{++} ions participate in the initiation of the coagulation process. This may be prevented by prompt adsorption of prothrombin and other clotting components onto $BaSO_4$¶(7,8). As with resin-collected plasma, zymosan adsorption of properdin may then be successfully applied. Details of the technic are given in the flow sheet (Table I). It is to be noted that the $BaSO_4$ adsorbed plasma is considered to be devoid of free Mg^{++} ions and is, therefore, made 10^{-3} M in Mg^{++} ions before incubation with zymosan. In 4 runs, in which the ACD-collected plasma had an average properdin activity of 4 units per ml,‖ less than 1 unit per ml of properdin remained in the plasma after zymosan adsorption. C. Table I presents a flow sheet of the technic employed for isolation of properdin from both types of plasma in our laboratory. The essence of the method remains the same as originally presented by Pillemer *et al.* The few modifications are noted. (1) *Temperature of incubation for adsorption of properdin on Zymosan*: The temperature at which properdin has been adsorbed onto zymosan

§ Zymosan from lots 5B171 and 6B14 has been purchased from Fleischman Yeast Division, Standard Brands, Inc.

‖ It has been found difficult for technical reasons to obtain accurate properdin determinations in plasma, particularly in ACD plasma. The properdin values given for plasma before zymosan adsorption should be considered to represent minimal values.

¶ Baker, C. P. $BaSO_4$ has been found superior to other brands for this purpose.

TABLE I



*Further concentration of properdin activity can be achieved by high speed centrifugation in the Spinco preparative ultracentrifuge (6)

is 15°C. (2) *Adjustment of pH*: Acetate buffer (pH 4, 0.2 ionic strength) has been substituted for 1.0 N hydrochloric acid for adjustment of the plasma to pH 6.9 prior to addition of zymosan. Local concentration of hydrogen ions which might produce irreversible alteration of plasma proteins is less likely to ensue from the use of acetic acid rather than from completely ionized hydrochloric acid. The mixture may, alternatively, be adjusted to pH 6.9 by bubbling CO_2 through it. This procedure also avoids the use of strong acids but has the disadvantages of being time consuming and of possible surface denaturation of protein by the stream of bubbles. As indicated by Pillemer *et al.*, the pH of the zymosan-plasma mixture rises during the hour's incubation at 15°C(1,5). We have found this rise in pH to be due to loss of carbon dioxide during stirring. Maintenance of constant pH 6.9 has been obtained by imposing a layer of carbon dioxide over the mixture while the pH is monitored by an automatic titrator.** (3) *Elution volume*: It has been found that a volume of buffer equal to 1/8th the volume of original plasma is adequate for elution of properdin from the zymosan-properdin complex.†† The buffer is made by mixing 0.09 plasma volume of veronal buffer (pH 7.4, 0.19 ionic strength) with 0.035 plasma volume of 2 M NaCl solution. The buffer is warmed to 37°C before suspension of the washed zymosan-properdin complex(5). (4) *Precipitation of properdin by zinc ions*: Properdin has been found to form a water-insoluble zinc complex. Thus when zinc glycinate reagent was added directly to the eluate to a final zinc concentration of 25 mM at pH 7.1-7.5, quantitative precipitation of zinc-properdin occurred. The precipitated properdin complex may be dissolved in small volume in the presence of ethylene-diamine-tetra-acetic acid (EDTA), thus greatly reducing the volume of fluid which must be dialyzed with a concomitant reduction in time of dialysis. The volumes suggested in the flow sheet result in solution of the properdin-zinc complex at 1/50 plasma volume. In large scale fractionation, where plasma volumes may be as great as 300 to 1000 liters of plasma, this solution may be accomplished satisfactorily at 1/500 plasma volume. (5) *Solubility of properdin in water*: After dialysis is completed, the dialyzed material is adjusted to pH 5.6 by careful addition of acetate buffer. Elevation of the ionic strength of the mixture must be avoided insofar as possible. Qualitative observations indicate that, in water, the pH of minimum solubility of properdin is close to 5.6(9). This is highly suggestive that the iso-electric point of properdin is near this pH value. Addition of a small amount of salt to the precipitated complex will dissolve the properdin at pH 5.6, showing the protein to exhibit the classical euglobulin behavior.

Results. Comparison of resin-collected and ACD plasma as sources of properdin. In

** Radiometer Titrator manufactured by Radiometer Co., Copenhagen, Denmark.

†† E. W. Todd and L. Pillemer, to be published.

TABLE II. Comparative Properdin Recovery from ACD and Resin Plasma Prior to Ultracentrifugation.

Age of plasma	Units of properdin recovered/ml of original plasma: ACD plasma	Resin plasma
1 to 2 days	2.18 (2 runs)	2.45 (11 runs)
1 to 2 wk	1.05 (2 ")	1.63 (7 ")
1 mo or more		.71 (3 ")

Table II, it is evident that properdin may be prepared from both fresh ACD and fresh resin-collected plasma although recoveries were somewhat better when the source material was resin-collected. Establishment of the feasibility of ACD plasma as a source of properdin is important, however, for this is the form in which essentially all of the human plasma available for fractionation occurs. It has been found difficult for technical reasons to obtain accurate properdin determinations in plasma, particularly in ACD plasma. The values obtained are often lower than the actual titer as witnessed by the eventual recovery of more than the original amount of properdin as calculated from the plasma value. To avoid confusion from this source, recovery of properdin activity is expressed (in Table II and other tables) as units recovered per milliliter of starting plasma, assuming that original pooled plasma properdin titers will vary only within a limited degree.

Age of plasma as a factor in properdin yields. Although human plasma should be fractionated promptly upon collection for optimal results, a major source of plasma for fractionation is from outdated blood (3 weeks or more after collection) from hospital blood banks. It has been shown that albumin and gamma globulin can be satisfactorily prepared from such plasma(10,11). Table II presents the recovery of properdin from plasma of varying age. The decrease of yield with the age of the plasma is undoubtedly due, at least in part, to the loss of plasma factors essential for properdin adsorption on zymosan. Some of the components of complement are known to become inactive during plasma storage. Nevertheless sufficient properdin can be recovered from outdated plasma to suggest that this major plasma source can be of value for large scale preparation of properdin.

Comparison of properdin recovery following zinc precipitation and following dialysis of the total eluate. From Table III, it is evident that essentially identical recovery of properdin was obtained whether or not the original eluate was dialyzed *in toto* or whether advantage was taken of the zinc precipitability of properdin to reduce the volume to be dialyzed.

Recovery of properdin in fraction I. Cold ethanol Method 6 of Cohn and collaborators (12) has been used in a preliminary study of the feasibility of preparing properdin by direct plasma fractionation without recourse to the use of zymosan. The major portion of the properdin activity is to be found in Cohn Fraction I when this fraction is precipitated at pH 6.9 or lower. This has proved true in 7 consecutive runs both for fresh resin-collected plasma and for plasma from outdated ACD blood. In 2 experiments designed to isolate properdin activity, 90-95% of the total properdin activity of the original plasma was recovered in a subfraction of Fraction I. Eventual recovery of properdin by direct fractionation appears extremely hopeful, thus eliminating the need for the costly and variable zymosan, except for assay purposes. Recovery of properdin from outdated plasma by direct fractionation will presumably not be dependent on the labile plasma components essential for the interaction of properdin with zymosan.

Pillemer has reported(1) the major properdin activity to be localized in Fraction III prepared from serum by the method of Deutsch(13). In our laboratory, as well, the

TABLE III. Comparison of Properdin Yields by Dialysis Procedure and by Zinc Precipitation Procedure Prior to Ultracentrifugation.

Run	Units of properdin recovered per ml of original plasma: Dialysis procedure	Zinc precipitation procedure
323 P	3.0	2.1
327	2.5	1.1
328	2.1	2.5
329	3.7	4.7
330	2.5	2.6
HP-2	.5	.6
331	2.5	3.5
Avg	2.4	2.4

properdin activity not found in Fraction I appears in Fraction III. Careful definition of the variables responsible for the distribution of properdin during fractionation of plasma is being pursued.

Summary. Methods have been presented for the preparation of properdin from resin-collected and from ACD plasma by the application of the zymosan adsorption technic of Pillemer *et al.* Preliminary data are presented suggesting the feasibility of the isolation of properdin from Cohn Fraction I without the use of zymosan.

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Some Factors that Influence Utilization of Antihemophilic Activity During Clotting.* (23454)

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When normal blood or plasma clots, it loses its antihemophilic activity(1). If plasma is first adsorbed with $BaSO_4$, antihemophilic factor (AHF) remains in the adsorbed plasma even after recalcification; it thus is stabilized by the adsorption procedure. This paper describes the effect on AHF utilization of adding various plasma and serum preparations and derivatives to this stabilized plasma. It was found that only when a source of thrombin was added would AHF disappear. Within the lower range of effective thrombin concentrations, the rate of loss of AHF was proportional to the amount of thrombin available. Preliminary reports of some of these data have been made previously(2).

Materials and methods. Oxalated plasma and *native, platelet-poor plasma* were prepared as previously described(1). *PTC-deficient plasma* was obtained from a patient with a moderately severe disease characterized by hemarthrosis and hematuria. *Plasma deficient in Stuart factor* was from the patient described by Hougie and associates(3). *"Dicumarol plasma"* was prepared from a dog to whom 1400 mg dicumarol had been administered over a 13-day period. *"Stabilized plasma"* was made by twice adsorbing oxalated plasma with $BaSO_4$ (Merck R; 100 mg/ml at 28°C for 20 min). *Normal and hemophilic canine sera* were obtained from blood allowed to stand in glass for 24 hours. *Serum accelerator factor (SAF)* was prepared as described earlier(4). *Plasma thromboplas-*

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† Markle Scholar in Medical Science.