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Human Plasma P Component: Isolation and Characterization[†,‡]

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ABSTRACT: A calcium-binding glycoprotein was isolated from human plasma by barium citrate adsorption-elution, DEAE-Sephadex chromatography, and chromatography on heparin-agarose in the presence of calcium. The preparation was homogeneous by disc gel electrophoresis, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, immunoelectrophoresis, and amino-terminal sequence to 30 residues. The subunit molecular weight estimate on sodium dodecyl sulfate-10% polyacrylamide gels was 25 000. Electrophoretic migration at pH 8.6 was with α-globulins when run in EDTA, but in calcium there was little, if any, migration from the origin. Amino acid analysis revealed two half-cystine residues, which formed an intrachain disulfide bond, a single methionine residue, and no γ-carboxyglutamic acid. The protein contained 11.2% carbohydrate consisting of 5.6% neutral sugars, 3.0% glucosamine and 2.6% sialic acid. Two fragments were isolated following cyanogen bromide digestion and gel filtration. The half-cystine residues and carbohydrate were present in the amino-terminal, larger fragment. Compared with vitamin K dependent proteins, the intact protein bound less tightly to DEAE and more tightly to heparin-agarose. The subunit size, composition, and amino-terminal sequence for this calcium-binding plasma protein are the same as described for P component purified from human serum and the sequence is nearly identical with that of P component from amyloid tissues. The amino-terminal region of P component from human plasma is homologous with the corresponding portion of C-reactive protein as 18 of the first 30 amino acid residues are identical; size and composition of cyanogen bromide fragments indicate internal homology as well. Of residues 8–12, four are identical with those in a five residue segment near the amino terminus of the Fc fragment of the γ_1-immunoglobulin chain. Plasma P component, however, failed to cross-react with either anti-C-reactive protein or anti-Fc fragment antibodies. A monospecific rabbit antibody to plasma P component was used in a quantitative immunoelectrophoretic system. The plasma level of a pool of normal donors was 44 μg per mL. Plasma P component was identical with a soluble fraction of P component from the tissue of a patient with amyloidosis when analyzed by immunodiffusion and tandem crossed immunoelectrophoresis. Subunit migration was identical in sodium dodecyl sulfate gel electrophoresis indicating that the plasma and tissue-derived forms have the same molecular size.

Amyloid is a pathological, proteinaceous substance which is associated with organ dysfunction. Extracts contain a fibrillar protein (either AA or immunoglobulin types, Benditt & Eriksen, 1971; Glenner et al., 1971) and a more soluble "P component" (Cathcart et al., 1965). The latter is so named because of a characteristic pentameric structure on electron micrographs (Bladen et al., 1966). Although the amount in saline tissue extracts from different patients varies, the presence of P component is independent of fibrillar type.

P component is also present in normal serum where it migrates as an α-globulin (Cathcart et al., 1967; Binette et al., 1974). When purified from normal human serum by gel filtration and preparative gel electrophoresis, P component was found by Binette et al. (1974) to be immunologically identical to an α_1-glycoprotein purified on carboxymethylcellulose by Haupt et al. (1972). Purification from serum by affinity chromatography using an insolubilized antibody to tissue P component has also been achieved (Benson et al., 1976).

In fractionating the subcomponents of the first component of complement (by affinity chromatography on insolubilized IgG with calcium), P component was copurified and thought to be a 4th subcomponent, called C1t (Assimeh et al., 1974). Although highly purified, complement activity of these preparations was due to contaminant protein (Painter, 1977). The preparation, however, contained pentameric structures on electron microscopy (Pinteric et al., 1976) and was immunologically identical to tissue P component and to serum preparations obtained by adsorption to unsubstituted agarose in the presence of calcium (Pepys et al., 1977a).

P component subunits, which are noncovalently bound, have reported molecular weights of 23 000 (Pinteric et al., 1976) or 36 000 (Benson et al., 1976). In the latter study a similar amino acid composition between tissue and serum P component preparations was found, although no amino-terminal sequence could be obtained from their serum fractions. In contrast, Osmand et al. (1977) reported a single amino-terminal sequence from the serum P component preparation (C1t) which suggests the presence of identical subunits. Homology with the C-reactive protein was also noted.

During the course of purifying vitamin K dependent proteins from human plasma, a calcium-binding glycoprotein which

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[1] A preliminary report of these properties has been presented: Thompson, A. R. (1977) *Blood Suppl. 1, 50*, 284.

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is identical with P component was isolated. Whereas previous reports of P component properties have been based upon serum or tissue preparations of poorly defined purity or with demonstrated contaminant protein, this plasma preparation was homogeneous by gel electrophoresis, immunoelectrophoresis, and sequence analysis. Subunit structure of plasma P component was therefore characterized and compared with that of an amyloid tissue extract. Calcium-dependent behavior during purification and electrophoresis was also studied as these properties were qualitatively similar to those of the vitamin K dependent proteins.

Materials and Methods

Materials. Whole blood was collected in bags containing citrate-phosphate-dextrose (Fenwall Laboratories, Martingrove, Ill.). Following centrifugation to remove red cells and platelet concentrates, 5–10 mg of heparin was added per bag and the bags were quick frozen individually at −70 °C. Human plasma from 30 normal donors was drawn into 0.10 volume of 3.8% sodium citrate, pooled, and centrifuged to remove red blood cells and again at 40 000*g* for 20 min to prepare platelet poor plasma (Thompson, 1977).

Human prothrombin was eluted in the void volume during heparin–agarose chromatography (Thompson, 1977) and was further purified by an additional heparin–agarose column in the presence of 10 mM $CaCl_2$. Prothrombin appeared in the void volume and was thus freed of contaminating factor X. Tissue P component was a soluble fraction which had been extracted from the liver of a patient with pulmonary tuberculosis and AA type amyloidosis; it was provided by Drs. Earl P. Benditt and Nils Eriksen, Department of Pathology, University of Washington (case A-4, Benditt & Eriksen, 1971). Rabbit antibody to human IgG_1 Fc fragment was kindly provided by Dr. Mart Mannick, Division of Rheumatology, Department of Medicine, University of Washington. Anti-C-reactive protein was purchased as rabbit antibody from Behring Diagnostics, Somerville, N.J., and as a goat preparation from ICN Pharmaceuticals, Cleveland, Ohio. Tos-$PheCH_2Cl$[2]-treated trypsin was obtained from Worthington Biochemical Corp., Freehold, N.J.

The following materials were obtained from commercial sources as indicated: DEAE-Sephadex A-50, Sephadex G-25 (coarse) and G-50 (superfine), and Sepharose 4B, Pharmacia, Piscataway, N.J.; agarose, SeaKem, Rockland, Maine; Coomassie brilliant blue R-250, acrylamide, and bis(acrylamide), Bio-Rad, Richmond, Calif.; grade I heparin, *p*-hydroxymercuribenzoate, D(+)-galactose, D(+)-glucosamine, type IV *N*-acetylneuraminic acid, dithiothreitol, Sigma Chem. Co., St. Louis, Mo.; D(+)-mannose, Pfanstiehl Lab., Waukegan, Ill.; CNBr, J. T. Baker, Phillipsburg, N.J.; complete and incomplete Freund's adjuvants, Difco, Detroit, Mich. Unless indicated, other chemicals were reagent grade.

Preparatory Procedures. Plasma P component was purified by a modification of the procedure used for human factor IX (Thompson, 1977). $BaCl_2$ (15 g/L) was added to 1 or 3 L of plasma and the barium citrate precipitate collected by centrifugation, washed, and then eluted with one-third volume of 0.15 M sodium citrate (pH 7.4). The eluate was then fractionated at 4 °C by the addition of 176 g/L ammonium sulfate (30% saturation) and the precipitate removed by centrifugation. Additional ammonium sulfate was added (214 g/L of original volume, 60% saturation) and the precipitate recovered by centrifugation and dissolved in 10 mL of 10 mM benzamidine. The fraction was either filtered through a Sephadex G-25 column (2.5 × 25 cm) equilibrated with 50 mM sodium citrate (pH 7.4) or dialyzed overnight against 100 volumes of the same buffer. The sample was then applied to a column of DEAE-Sephadex (4 × 22 cm) and washed with the initial buffer before beginning an exponential gradient from 50 to 150 mM sodium citrate (pH 7.4) containing 1 mM benzamidine as previously described (Thompson, 1977); 20-mL fractions were collected. Further details are in the legend to Figure 1. Peak fractions were pooled and either precipitated by 60% ammonium sulfate, redissolved, and dialyzed or simply dialyzed against 0.1 M NaCl-50 mM Tris (pH 7.5). $CaCl_2$ was then added to a final concentration of 1 mM. The preparations were divided to avoid overloading on the heparin–agarose column. A sample equivalent to 0.5 L starting plasma was passed through a heparin–agarose column (2.5 × 6 cm) which had been prepared as previously described (Fujikawa et al., 1973). The column was washed with 100 mL of 50 mM Tris (pH 7.5) containing 0.3 M NaCl and then 100 mL of 50 mM Tris (pH 7.5) containing 0.5 M NaCl. P component was then eluted with 0.15 M sodium citrate (pH 7.4). Alternatively, the binding of DEAE-purified P component to unsubstituted Sepharose 4B was compared with the binding to heparin–agarose on 2.5 × 4 cm columns.

Protein concentration was determined by absorbance at 280 nm using an extinction coefficient $E_{1cm}^{1\%} = 18.2$ as reported by Haupt et al. (1972), or by the biuret method as modified by Yatzidis (1977) using a human albumin standard (Dade, Miami, Fla.). Conductivity was determined on a Radiometer CDM2f meter.

Digests. Cyanogen bromide digestion of P component was performed as previously described (Thompson et al., 1977). P component (4.0 mg) was incubated with 25 mg of CNBr for 12–36 h in 80% formic acid (technical grade), then diluted 10-fold with distilled H_2O, and lyophilized. The digest was redissolved in 1 mL of 10% acetic acid and then fractionated on a Sephadex G-50 column equilibrated in 10% acetic acid (see legend to Figure 5 for details). Incubation with trypsin was carried out for 30 min at 37 °C in a volume of 0.1 mL of 50 mM Tris (pH 7.8) containing either 2 or 10 μg of Tos-$PheCH_2Cl$-treated trypsin and 100 μg of P component (molar ratios 1:50 and 1:10). Samples were diluted, reduced, and electrophoresed as described below.

Electrophoretic Techniques. Alkaline disc gels were performed as described (Thompson, 1977). Polyacrylamide gel electrophoresis in 1% $NaDodSO_4$ was carried out according to Weber & Osborn (1969) using the buffer modification and (where applicable) the reducing conditions of Fairbanks et al. (1971). Polyacrylamide concentrations were either 7.5 or 10%; for the latter, some experiments utilized "double-cross-linked gels" in which the concentration of bis(acrylamide) was doubled. Protein was stained with Coomassie brilliant blue or periodic acid–Schiff carbohydrate reagent as previously described (Thompson et al., 1977). For molecular weight estimates, the following standards were used (molecular weight and mobility relative to marker dye provided in parentheses for representative results using 83-mm 10% polyacrylamide gels): ovalbumin (44 000, 0.25); carbonic anhydrase (29 000, 0.50); myoglobin (17 700, 0.78); lysozyme (14 600, 0.88). Electrophoresis in 1% agarose, with either 2 mM EDTA or 2 mM calcium lactate, was performed on coated 5 × 5 cm plates in 75 mM barbital (pH 8.6) as described by Stenflo & Ganrot (1972).

Immunologic Methods. White female rabbits were im-

[2] Abbreviations used: DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid (Na_2 salt); $NaDodSO_4$, sodium dodecyl sulfate; Tos-$PheCH_2Cl$, L-1-tosylamido-2-phenylethyl chloromethyl ketone; Tris, tris(hydroxymethyl)aminomethane.

TABLE I: Purification of Human Plasma P Component.

purification step	vol (mL)	absorbance (total units), 280 nm	P component (total mg)[a]	yield (%)	purification (fold)
plasma	1000	50 000[b]	49	100	
barium citrate	500		45	92	
ammonium sulfate (30–60%)	28	224[b]	39	80	178
DEAE-Sephadex	150	39	23	47	600
heparin agarose	5	25	15	31	620

[a] Determined by quantitative immunoelectrophoresis and compared with a simultaneous standard of isolated P component for which concentration was calculated by extinction. [b] To minimize the effects of turbidity at these steps, absorbance measurements were performed on aliquots diluted in 1 M acetic acid.

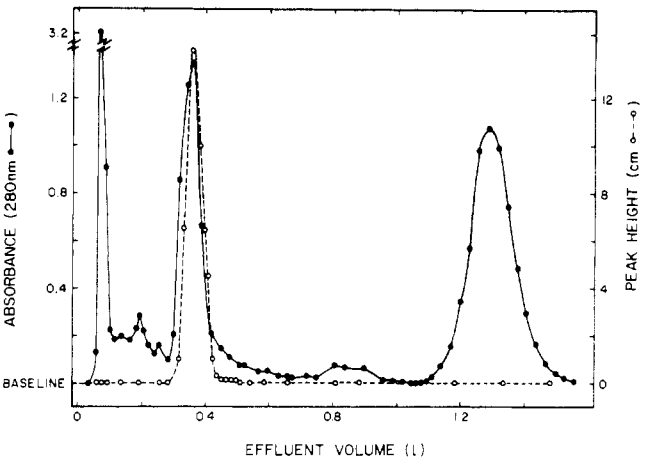

FIGURE 1: DEAE-Sephadex chromatography of barium citrate eluate. A 4 × 22 cm column was equilibrated with 50 mM sodium citrate (pH 7.4) containing 1 mM benzamidine. Ammonium sulfate fractionated eluate (50 mL) was added and the column washed with an additional 50 mL of starting buffer. The exponential gradient to 0.15 M sodium citrate was begun at that point. Effluent volume corresponds to the volume collected after application of the sample. P component concentration was monitored by quantitative immunoelectrophoresis (open circles and dashed lines).

munized with 0.5–1 mg of P component prepared by either DEAE chromatography or citrate elution from heparin–agarose. Antigen was emulsified in an equal volume of Freund's adjuvant and injected intradermally using multiple sites on the animal's back. Booster shots were administered 1–6 months later with venous blood collected by ear-vein bleeding 7–10 days following the boost; blood was clotted in 1 × 15 cm tubes and the sera centrifuged. Antisera was stored at −20 °C with 0.01% NaN_3. Immunodiffusion and immunoelectrophoresis were performed as described (Thompson, 1977) using agarose-coated slides, 1% agarose in 40 mM barbital buffer (pH 8.6) and 2 mM EDTA. Quantitative immunoelectrophoresis (Laurell, 1966) and crossed immunoelectrophoresis were performed as described by Weeke (1973) using agarose-coated 5 × 5 cm plates to which 1% agarose in barbital buffer containing 2 mM EDTA and 0.2–1.0% rabbit anti-P component (in the final dimension) was applied. Tandem crossed immunoelectrophoresis utilized a modification described by Kroll (1973). These procedures were carried out on a LKB 2177 Multiphor apparatus, cooled to 23 °C.

Analytical Methods. Amino acid compositions were determined by AAA Laboratories, Seattle, Wash. on a Durrum Model D-500 analyzer. After acid hydrolysis, analyses (see Table II) were performed as previously described (Thompson et al., 1977). For γ-carboxyglutamic acid analysis, Aminex A-27 resin was used as described by Tabor & Tabor (1977) being run by AAA Laboratories on a Beckman Model 120B analyzer with a cell pathlength of 20 mm. A synthetic γ-carboxyglutamic acid was used as a standard; alkaline hydrolysates of proteins were analyzed. Carbohydrate analyses were performed using methods as previously described (Thompson et al., 1977) on two separate preparations of P component which had been dialyzed extensively against 10% acetic acid, lyophilized, weighed, and reconstituted at 1.0 mg/mL in 25 mM NaCl–25 mM Tris (pH 7.5) containing 0.5% $NaDodSO_4$. Triplicate determinations were run on each preparation and values reported are the averages from the two preparations. Procedures used were as follows: neutral sugars by the Orcinol and phenol–sulfuric acid methods; hexosamine by the Elson-Morgan method and on the Durrum analyzer (AAA Laboratories); sialic acid by the thiobarbituric assay. Titration for free sulfhydryl groups was performed spectrophotometrically with *p*-hydroxymercuribenzoate in acetate (pH 4.6) containing 2% $NaDodSO_4$ as described by Glazer et al. (1975). Automated sequence analysis was carried out on a Beckman Sequencer, Model 890B, using methods previously described (Thompson et al., 1977). Phenylthiohydantoin-amino acid derivatives were identified by high pressure liquid chromatography on a modified Durrum analyzer (Ericsson et al., 1977) or on a Waters Associates system, Model 6000A (Brigden et al., 1976).

Results

Purification. A representative purification of P component from 1 L of plasma is presented in Table I. The overall yield by quantitative immunoelectrophoresis was 31%. Following barium citrate adsorption, less than 5% of the total immunoreactive P component remained in the supernatant. After ammonium sulfate fractionation of the barium citrate elutate, P component could not be identified in the 30% precipitate and, generally, less than 10% was present in the 60% ammonium sulfate supernatant.[3] On DEAE-Sephadex, P component trailed within the second major protein peak (0.4 L) corresponding to 60 mM citrate by conductivity (Figure 1). The last major protein peak, beginning around 1.2 L (approximately 140 mM citrate), contained the vitamin K dependent clotting factors. Pooled DEAE-purified P component from 3-L plasma preparations contained sufficient P component to "overload" the heparin–agarose column (see Materials and Methods). Thus, the larger preparations were divided into fifths and the smaller (1-L plasma preparations) into halves or thirds and run separately over heparin–agarose as described in Materials and Methods. All analyses were performed on fractions eluted from heparin–agarose by citrate.

Preliminary experiments demonstrated that the binding to heparin–agarose was calcium dependent. To compare the binding of P component to heparin–agarose with the binding to agarose alone, DEAE-purified material from the equivalent of 0.3 L starting plasma was passed over columns of each of these two gels. The columns had been equilibrated with 50 mM NaCl–50 mM Tris (pH 7.5) containing 1 mM $CaCl_2$. For agarose alone, under these conditions, nearly all the protein (>95% absorbance and P component) was in the void volume and none in 0.3 or 0.5 M NaCl washes. The citrate eluate from

[3] For some preparations with lipemic starting plasma, however, numerous floating particles were observed in the 60% ammonium sulfate supernatant. By quantitative immunoelectrophoresis, the particulate material contained up to approximately 10% of the total amount of P component.

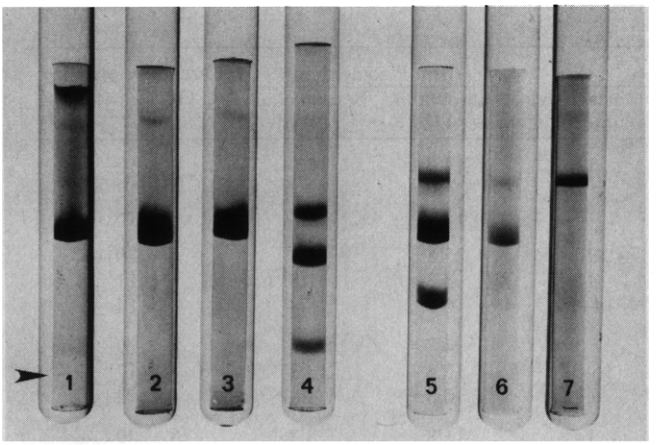

FIGURE 2: Sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis of plasma P component. Anode is at the bottom and the marker dye (bromophenol blue) migrated to 90 mm on these gels (arrow). Gels were run under standard conditions (Weber & Osborn, 1969) except for 5–7 which were double cross-linked (see Materials and Methods). Gel 1 represents 30 μg of native P component diluted in $NaDodSO_4$ and applied directly; equal aliquots of the same sample were either heated at 37 °C for 30 min in 1% $NaDodSO_4$ (gel 2) or reduced overnight at 37 °C with dithiothreitol (gel 3). Gels 4 and 5 represent different preparations of P component digested with CNBr for 12 and 36 h, respectively. Gel 6 was the same sample as gel 5 but stained for carbohydrate. Gel 7 represented a sample of 20 μg of tissue P component. Except for gel 6, staining was with Coomassie brilliant blue.

unsubstituted agarose contained no more than 5% of the starting P component protein. In contrast, essentially all of the protein from the DEAE-purified preparation bound to the heparin–agarose; approximately 20% of the absorbance units were eluted in the 0.3 M NaCl wash and an additional 5% in the 0.5 M NaCl wash. Neither of these wash fractions contained detectable P component by quantitative immunoelectrophoresis. P component was quantitatively recovered in the citrate eluate. Results identical with the heparin–agarose experiment were also obtained when the void volume from the unsubstituted agarose was passed over a heparin–agarose column.

Purity of the Preparation. A single band was seen on alkaline–urea disc gels (not shown) and on 10% polyacrylamide gels with $NaDodSO_4$ (Figure 2). When the sample was diluted but not heated in $NaDodSO_4$ prior to application to the gel, a second band corresponding to a multimer with a molecular weight in excess of 100 000 was observed (gel 1). Following incubation in $NaDodSO_4$ at 37 °C for 30 min (or boiling for 1 min), only the single band of the subunit size was observed (gel 2). There was no further change of subunit size upon dithiothreitol incubation (gel 3). Molecular weight estimate for P component on 10% polyacrylamide gels in $NaDodSO_4$ was 25 000; relative mobility on the representative run (see Materials and Methods) was 0.55. A value of 23 000 was found with the same standards on 7.5% gels, and 27 000 was observed on 10% polyacrylamide gels containing a doubled concentration of bis(acrylamide). The intact subunit stained positively for carbohydrate (not shown). P component was also homogeneous by immunoelectrophoretic techniques. Rabbit antibody raised against heparin–agarose-purified P component demonstrated a single arc when run with either 5 μg of heparin–agarose- or 20 μg of DEAE-purified preparations (not shown). When the same P component preparations were run against rabbit antibody raised against DEAE-purified P component, the heparin–agarose preparation again gave a single arc whereas the DEAE preparation gave 3–4 additional arcs (not shown). On quantitative immunoelectrophoresis

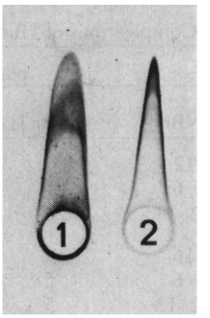

FIGURE 3: Quantitative immunoelectrophoresis of human plasma P component in agarose. Well 1 contained 10 μL of normal human plasma pool and well 2 contained 10 μL of isolated P component (44 μg). Agarose contained 1% monospecific anti-P-component sera. Anode is at the top; 5 V/cm was applied for 3 h. Height on original plate measured 15 mm.

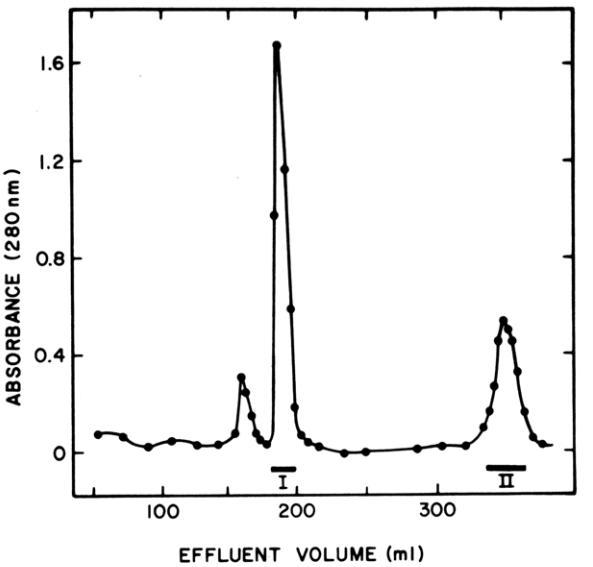

FIGURE 4: Fractionation of CNBr fragments on Sephadex G-50. A 2.5 × 98 cm column was run at 20 mL/h; 5-mL fractions were collected and read in a Beckman DU spectrophotometer at 280 nm. CNBr fragments I and II were recovered by lyophilization of pooled fractions as indicated by the horizontal bars.

(Figure 3), a single peak was observed when the monospecific antibody was employed. The latter was used to quantitate the amount of protein in a normal plasma pool. Compared with dilutions of heparin–agarose purified P component, the plasma contained 44 μg/mL.[4] When P component protein concentration was calculated from the biuret results instead of by extinction, the level was 1.4-fold higher. When the antibody to DEAE-purified P component was substituted in the quantitative immunoelectrophoretic system, six distinct peaks were observed when a barium citrate eluate was run, whereas a single peak was formed by heparin–agarose-purified preparations (not shown).

Electrophoresis in Agarose. The relative electrophoretic mobilities of human P component and human prothrombin (20 μg each) were compared at pH 8.6 in EDTA vs. calcium. Migration in EDTA was that of an α-globulin whereas the proteins' migrations were markedly retarded by running in Ca^{2+}. When the P-component sample was equilibrated with calcium prior to running, no migration from the well was observed on electrophoresis in calcium.

[4] The value was based upon relative height; the use of area would give a somewhat higher normal plasma level.

TABLE II: Amino Acid Compositions of Human Plasma P Component.[a]

amino acid	plasma P component[b] CNBr I	CNBr II	sum (I + II)	whole protein	nearest integer	serum P component[c] Haupt et al. (1972)	Benson et al. (1976)	C-reactive protein[d] Oliveira et al. (1977)
Asx	12	5	17	15.3	15	14.7	15.0	15
Thr	6	1	7	7.2	7	6.0	12.2	12
Ser	12–13	2–3	15	14.9	15	11.1	20.2	18
Glx	16	5	21	20.0	20	25.8	21.1	20
Pro	6	5	11	11.0	11	8.3	11.8	11
Gly	12–13	5	17–18	14.5	14–15	6.7	18.4	14
Ala	5–6	3	8–9	7.5	7–8	4.7	10.5	9
Val	12–13	4	16–17	16.5	16–17	13.4	13.3	17
Met	1[e]	0	1	1.0	1	0.8	1.8	2
Ile	7	4	11	11.0	11	11.8	5.3	9
Leu	14	6	20	18.1	18	17.6	12.9	14
Tyr	8	3	11	10.6	11	15.9	6.1	7
Phe	9	0	9	9.0	9	11.3	7.0	12
His	3	0	3	3.2	3	4.0	4.4	2
Lys	8	1	9	9.5	9–10	11.2	10.4	12
Arg	7	1[f]	8	7.8	8	10.1	6.5	6
½-cystine	2[f]	0	2	1.7[f]	2	1.0	3.4	2
Trp	ND	ND		4.4[g]	4	7.5		5
total	140–144	45–46	186–189		181–184			187

[a] Compositions are expressed as residues/subunit. [b] Two preparations of CNBr I were acid hydrolyzed for 24 h and the results averaged. The values for CNBr II represent a 24-h hydrolysis of a single preparation. For the whole protein, duplicate samples were hydrolyzed for 24, 48, 72, and 96 h; values are averaged from duplicate determinations of each hydrolysis except for Thr and Ser which were extrapolated to zero time and Val and Ile which are the 96-h values. [c] The composition of Haupt et al. (1972) was adjusted to 184 residues; values from Benson et al. (1976) were averaged from their results of 24-h hydrolysates of two separate serum preparations and adjusted to 180 residues as Trp was not determined. [d] Calculated from sequence. [e] Determined as homoserine (1.0 residues). [f] Determined as cysteic acid (Hirs, 1967); the value for CNBr I was 1.6 residues. [g] Determined after alkaline hydrolysis (Hugli & Moore, 1972).

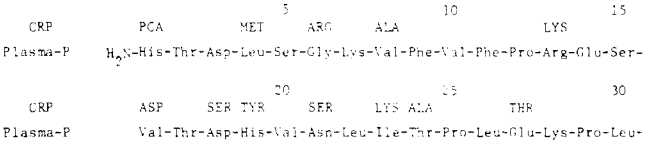

FIGURE 5: Amino-terminal sequence of plasma P component. Residue number assignment and sequence of C-reactive protein are taken from Oliveira et al. (1977). Where C-reactive protein differs from P component sequence, the former residues are indicated in capital letters above the corresponding P component residue.

Digestion of P Component. Following CNBr digestion, two smaller protein bands were seen on $NaDodSO_4$–polyacrylamide gels (Figure 2, gels 4 and 5). The larger of these had an estimated molecular weight of 19 000 (relative mobility was 0.72 on the representative run; see Materials and Methods) and contained the carbohydrate (Figure 2, gel 6). Cyanogen bromide fragments were fractionated by filtration through G-50 Sephadex (Figure 4). Electrophoresis in $NaDodSO_4$–10% polyacrylamide double cross-linked gels gave single bands for fragments I and II. A variable amount of protein migrating as intact subunit was seen in two of three preparations (upper band, Figure 2, gels 4 and 5; first peak, Figure 5). After lyophilization the initial G-50 peaks contained insufficient material for analysis. In a separate experiment, isolated CNBr fragment I did not bind to heparin–agarose in the presence of 1 mM calcium.

Following incubation with trypsin at 1:50 or even 1:10 molar ratios, the migration of dithiothreitol-reduced P component subunit in $NaDodSO_4$–10% polyacrylamide gels was unchanged (not shown).

Analyses. The amino acid composition of P component is presented in Table II. The two half-cystine residues were disulfide linked as native P component failed to react with *p*-hydroxymercuribenzoate in the presence of $NaDodSO_4$. Amino acid analysis of the CNBr fragments demonstrated that the larger one contained two half-cystines and 140–144 residues, without considering tryptophan. This fragment originated from the amino-terminal end of the intact protein as indicated by its single residue of homoserine (Table II). The smaller fragment contained only two basic residues and no homoserine or phenylalanine.

An analysis for γ-carboxyglutamic acid in P component was negative. Whereas this residue was readily identified from a hydrolysate of human prothrombin (7 nmol), a hydrolysate of P component (17 nmol) showed no change of absorbance at the elution position of γ-carboxyglutamic acid standard. Since prothrombin contains ten residues per molecule (Magnusson et al., 1974), four residues per subunit of P component would be expected to give an absorbance peak of approximately the same height as the prothrombin and one residue per molecule should have been easily detectable in this system.

Carbohydrate composition was as follows: neutral sugars, 5.6% by the Orcinol method and 6.8% by phenol–sulfuric acid (vs. 1:1 galactose:mannose standard); hexosamine 3.0% by Elson-Morgan and 2.8% by amino acid analysis where the peak was identified as glucosamine; sialic acid, 2.6%. Although somewhat less precise, the Orcinol result was used in calculating total carbohydrate (11.2% by weight) because of less variability between the duplicate samples.

Amino-Terminal Sequence. The first 30 amino-terminal residues were identified and are presented in Figure 5. For the amino-terminal position, a trace amount (less than 10%) of glycine was observed on two determinations in addition to the

histidine; the subsequent 29 residues each had single identifiable amino acid derivatives. The initial yield was 70%; average stepwise yield through the second histidine (residue 19) was 90%. The larger CNBr fragment was also sequenced through six residues and these were identical with the amino-terminal analysis of the whole protein.

Identity of Plasma and Tissue P Components. The tissue P component preparation gave a protein band on Na-DodSO$_4$–10% polyacrylamide double cross-linked gels which migrated identically with plasma P component (Figure 2, gel 7). Furthermore, equal mixtures of these two preparations gave the same single band (not shown). Figure 6 shows the reaction of complete identity on (A) immunodiffusion and (B) tandem crossed immunoelectrophoresis between tissue and plasma P components. Plasma P component failed to show any precipitation reaction to the two commercial preparations of anti-C-reactive protein or the anti-Fc fragment antibody (Figure 6A). The anti-C-reactive protein antibodies failed to show precipitin bands against the normal plasma pool (not shown) but gave strong lines to the plasma of a patient with a high erythrocyte sedimentation rate in which case the precipitin lines showed complete nonidentity (crossing) with anti-P component (Figure 6A, well 5 vs. antibodies in wells 4, 6, and a).

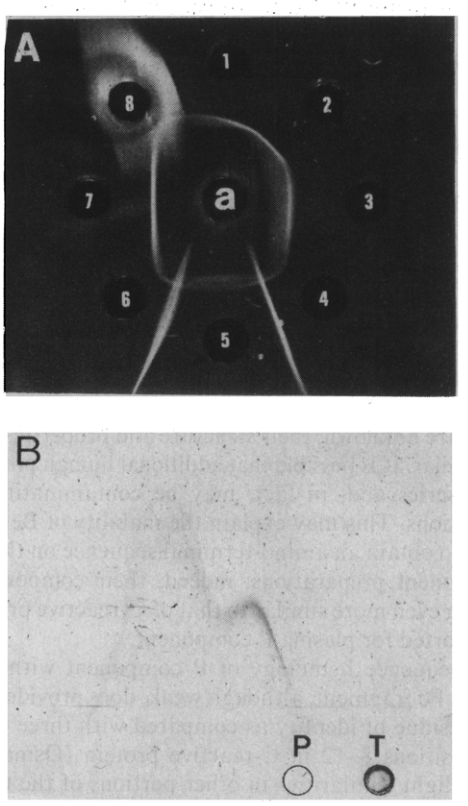

FIGURE 6: Immunologic comparison of plasma and tissue P components. (A) Immunodiffusion in 0.5% agarose (unstained). Center well contains 10 μL of monospecific rabbit anti-P component antibody (a) and 10-μL samples were applied to the surrounding wells as follows: (1) normal human plasma pool; (2) tissue P component (approximately 5 μg); (3 and 7) isolated plasma P component (5 μg); (4 and 6) different commercial preparations of rabbit and goat anti-C-reactive protein (respectively); (5) plasma from a patient with an erythrocyte sedimentation rate of 100 mm/h whose plasma reacted with anti-C-reactive protein; and (8) rabbit antisera to the Fc fragment of the γ chain of IgG$_1$. (B) Tandem crossed immunoelectrophoresis. Well P contained 10 μg of isolated P component, and well T approximately 5 μg of the tissue preparation. After diffusing 30 min, the wells were evacuated and refilled with 10 μL of agarose (Kroll, 1973). Electrophoresis was then carried out at 5 V/cm for 1 h with the anode to the left. The upper two-thirds of the agarose was then removed and substituted with 1% agarose containing 1% monospecific anti-P component and the second electrophoresis carried out with the anode at the top as in Figure 3. Washed slabs were stained with Coomassie brilliant blue, destained, and then dried.

Discussion

P component has been isolated from human plasma[5] and is homogeneous by gel electrophoresis, immunoelectrophoresis, crossed immunoelectrophoresis and amino-terminal sequence analysis. Compared with previous serum and tissue preparations, the subunit molecular weight of this glycoprotein on polyacrylamide gels was in good agreement with the 23 000 determined by sedimentation equilibrium (Pinteric et al., 1976) but was significantly lower than the 36 000 estimated by gel electrophoresis for serum and tissue preparations by Benson et al. (1976). On amino acid analysis, major differences in composition were noted for at least seven residues compared with each of the previous reports on the serum preparation (Table II). The carbohydrate content agreed closely with that determined by Haupt et al. (1972) on a serum P component and that by Glenner et al. (1968) on a tissue preparation. Binette et al. (1971), however, found a much lower content in their tissue preparation. Although Benson et al. (1976) found a blocked amino terminus on sequence analysis, the sequence data to 28 residues reported by Osmand et al. (1977) is identical with that determined here. Sequence of 20 of the first 23 residues from a tissue amyloid P component preparation (Skinner et al., 1974) indicated that only 11 of the 20 residues were identical with the plasma P component sequence presented here. More recently, eight of the first ten residues of two additional tissue amyloid P components have been sequenced (Skinner, et al., 1976); all eight of these positions are now identical with the plasma P component sequence. Levo et al. (1977) examined an additional tissue P component preparation and provided data on residues 3–18 and 20–27; these were identical with the plasma P component sequence. Their preparation, however, was one residue shorter and had no identifiable residue at the amino terminus (position 2). Thus evidence exists for microheterogeneity, particularly among preparations from patient tissue extracts. The criteria of homogeneity among previous preparations are less well-defined and for some (e.g. "Clt", Painter, 1977) contaminant proteins were present. In comparing the properties presented here with previous data, discrepancies may well be due to differences in the purity of the preparations.

Further evidence concerning the structure of P component subunit arises from its homology with the C-reactive protein which is striking despite the lack of immunologic cross-reactivity. The complete sequence of C-reactive protein has recently been presented (Oliveira et al., 1977) and the amino acid composition calculated from the sequence is quite similar to that presented here for plasma P component (Table II). For C-reactive protein, however, the amino terminus is blocked. Nevertheless, 18 of the first 30 residues are identical. One noteworthy difference is in the 4th position which is methionine in C-reactive protein but leucine in P component. The second methionine residue in the C-reactive protein is at position 142 of a total of 187. Assigning methionine to this position in P component is consistent with (1) the size of the CNBr frag-

[5] In the isolation of vitamin K dependent clotting proteins, heparin and benzamidine are added to minimize exposure to proteolysis. Previously reported preparations of P component have been from serum or, at least, thrombin-clotted, out-dated plasma. Indirect evidence for cleavage of P component (Clt) by *Crotalus etrox* (western diamond-back rattlesnake) venom (Minta et al., 1977) suggested a tryptic-like cleavage of this protein. The plasma P component preparation, however, was not hydrolyzed by trypsin in the present study.

ments observed from P component (the amino-terminal one being approximately 144 residues by our analysis); (2) the presence of one residue of homoserine in CNBr fragment I but none in fragment II; (3) the similarity of amino acid compositions of CNBr fragments with corresponding regions of C-reactive protein; and (4) the amino-terminal sequence of CNBr fragment I. Assuming further internal homology with the C-reactive protein in unsequenced regions, the half-cystine residues in CNBr fragment I could be placed at positions 36 and 78. The placement of carbohydrate can be speculated upon for C-reactive protein as the only permissive sequence (namely, Thr-X-X-Pro; Jolles et al., 1972) occurs within the disulfide loop of the major CNBr fragment.

Although the functions of P component and C-reactive protein are unknown, their structure and properties are strikingly similar. It is possible that additional human proteins exist in this series and, in fact, may be contaminating certain preparations. This may explain the inability of Benson et al. (1976) to obtain an amino-terminal sequence on their serum P component preparations; indeed, their composition is if anything even more similar to that of C-reactive protein than that reported for plasma P component.

The sequence homology of P component with immunoglobulin Fc fragment, although weak, does provide one additional residue of identity as compared with three of the five from positions 8–12 in C-reactive protein (Osmand et al., 1977); slight similarities in other portions of the C-reactive protein were also noted (Oliveira et al., 1977). The positions of the disulfide bond and possible carbohydrate attachment site constitute further gross homology, although γ chain carbohydrate is bound to an asparagine residue (Edelman, 1970). In the immunoglobulin type of amyloidosis, sequence of the fibrillar fraction corresponds to the variable region of the immunoglobulin light chain (Glenner et al., 1971). P component, however, is also present in amyloid tissues where the fibrillar component is AA (nonimmunoglobulin), making the P component in amyloid tissue independent of the fibrillar type.

During the course of preparation, the adsorption to barium citrate was similar to that of the vitamin K dependent clotting proteins. A variable amount of P component, however, bound to lipid components in the ammonium sulfate supernatant. These properties are reminiscent of C-reactive protein which can be adsorbed and eluted from barium sulfate (Ganrot & Kindmark, 1969) and precipitated with phospholipids in the presence of divalent ions (Hokama et al., 1974). Haupt et al. (1972) also found their P component (glycoprotein) preparation was adsorbed by $BaSO_4$ or calcium phosphate.

Electrophoretic mobility of P component in calcium was qualitatively similar to that of prothrombin. Boxer et al. (1977) suggested that P component was a vitamin K dependent protein as patients on warfarin had low or undetectable levels. On the contrary, we have recently evaluated samples from 24 patients on warfarin (Thompson, 1978) and found their levels to be normal. The absence of γ-carboxyglutamic acid residues demonstrates that, if vitamin K is involved in the binding of calcium to P component, it is through a different mechanism. At least some of the calcium-binding sites of P component constitute a difference from C-reactive protein as the former was removed from the latter by its calcium-dependent binding to agarose (Pepys et al., 1977b).

The variable amounts of P component in tissues from different patients with amyloidosis probably reflect the isolation techniques. In view of the calcium dependence in heparin–agarose binding, extraction by normal saline alone would probably be insufficient to release P component as it may well be calcium bound to tissue mucopolysaccharides, such as heparitin sulfate. The latter substance was isolated from amyloid infiltrated tissues (Linker et al., 1958); up to 2% by weight of defatted amyloid tissue is mucopolysaccharide (Dalferes et al., 1969). Glycoprotein material has been found associated with the heparitin extracted from amyloid (Dalferes et al., 1967) but composition data differs from intact, isolated P component. P component content of normal tissues needs to be reevaluated and related to sulfonated acid mucopolysaccharide content. Although the pathogenesis of amyloidosis is unknown, plasma P component could bind passively to mucopolysaccharides that had accumulated in altered tissues and thus contribute to organ dysfunction.

Because of the structural homology between P component and C-reactive protein and the presence of the latter as an "acute phase reactant", plasma P component levels in inflammatory disease states would be of interest. An additional question raised by the weak resemblance to immunoglobulin is whether or not P component is associated with or originates from plasma cells or B-lymphocytes (normal or malignant). The monospecific antibody can facilitate investigations of these questions.

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Sequence of a Rabbit Anti-*Micrococcus lysodeikticus* Antibody Light Chain†

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ABSTRACT: The complete sequence of rabbit antibody light-chain L 120 has been elucidated. The antibody was raised against *Micrococcus lysodeikticus* bacteria and is specific for the external part of the cell wall. All protein used in this work was obtained from a single 50-mL bleeding. The variable region of L 120 is compared to 13 other sequences of chains of different specificities. The constant region of this b4 κ chain is identical to that of two other constant regions published earlier. The general structure of the rabbit light chain is compatible with the three-dimensional folding proposed for human myeloma chains.

The elucidation of the structure-function relationship and of the genetic mechanisms involved in IgG synthesis relies on the comparison of the sequences of a large number of light and heavy chains from naturally raised antibodies for which the specificity is well established. While many myeloma human and mouse immunoglobulin antibody sequences have been reported, only a few primary structures of induced rabbit antibody molecules are available; mainly those of anti-pneumococcus type III and type VIII, anti-streptococcus antibodies (Chen et al., 1974; Jaton, 1974a,b, 1975; Margolies et al., 1975; Braun et al., 1976), and anti-*p*-azabenzoarsonate antibodies (Appella et al., 1973).

We report here the complete light-chain sequence of a rabbit antibody raised against *Micrococcus lysodeikticus*, a gram-positive bacterium. This antibody is specific for the external part of the *Micrococcus lysodeikticus* cell wall, which is composed of a polymer of glucose-*N*-acetylmannoseaminuronic acid. A comparison of the L 120 sequence with previously reported anti-pneumococcal, anti-streptococcal and anti-*p*-azabenzoarsonate light chains is discussed, and the existence

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