Fractionation and Characterization of Normal Rabbit Plasma Proteins

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1. An adaptation of the low-temperature low-salt ethanol procedure for the fractionation of rabbit plasma proteins into six fractions is described. 2. The composition of the fractions and the distribution of haptoglobins, caeruloplasmin and transferrin were determined. The protein and protein-bound carbohydrate distribution in the fractions is similar to that of human plasma proteins separated by a similar procedure. 3. The purification of albumin, α_1 -acid glycoprotein, transferrin and γ -globulin was carried out.

The pathogenesis of amyloidosis remains unknown, but increasing evidence suggests that the formation of the amyloid substance, in both clinical and experimental situations, may have a close relationship to changes in plasma proteins (Eklund & Reimann, 1936; Dick & Leiter, 1937; Snapper, Turner & Moscovitz, 1953; Richter, 1956; Giles & Calkins, 1958; Frensdorff, Sohar & Heller, 1961; Louhija & Miettinen, 1962). The availability of a well-established method for the induction of experimental amyloidosis in the rabbit provides an opportunity for an investigation of the alterations of serum proteins occurring during the development of amyloidosis in this species and of the relationship between these protein changes and the pathogenesis of this disease.

In the present paper the first part of this study is reported and the procedure of plasma-protein fractionation, the partial characterization of the proteins in the fractions obtained and the purification of some of the plasma proteins in the normal rabbit are described.

METHODS

Blood from mature normal New Zealand white rabbits (Pel Freez Biologicals Inc., Rogers, Ark., U.S.A.) was collected in acid-citrate-dextrose solution, pooled and separated into six fractions. Samples (10 and 20 ml.) were fractionated according to the procedure presented in Scheme 1. This procedure is a modification of a method previously employed to fractionate human plasma and synovial-fluid proteins (Schmid, Rosa & MacNair, 1956) and is based on method X of Cohn *et al.* (1950). The following changes were introduced to obtain satisfactory fractionation of the rabbit plasma proteins: (1) the amount of reagents used in all the various steps was decreased because of the slightly lower plasma-protein values in the rabbit; (2) the zinc acetate concentration employed in the precipitation of fractions IV+V was 0.017 M instead of 0.02 M; (3) the

pH of the reagent used in the extraction of fraction V from fractions IV+V was adjusted so that the pH value of the suspension was 4.90 instead of 6.03-6.06, and the concentration of zinc acetate was decreased to 4 mM.

The protein composition of the six fractions obtained by this procedure was examined by vertical starch-gel electrophoresis performed according to the method of Smithies (1959). The distribution of haptoglobins in the fractions was determined by the dianisidine method of Owen & Smith (1961). Caeruloplasmin was identified by the *p*-phenylene-diamine dihydrochloride method of Morell & Scheinberg (1960), as modified by McAlister, Martin & Benditt (1961), and by the dianisidine method of Owen & Smith (1961), with omission of the peroxide reagent. Transferrin was located by the technique described by Giblett, Hickman & Smithies (1959). α_1 -Acid glycoprotein was identified by comparison of its mobility in starch gel with a specimen of human α_1 -acid glycoprotein.

The relative concentration of the proteins in the plasma and the fractions was determined by horizontal paper electrophoresis in barbital-citrate buffer, pH 8.60 and *I* 0.1. The paper strips were stained with Amido Schwarz 10B and scanned in a Spinco model RB Analytrol.

The purification of albumin and transferrin from fraction V and γ -globulin from fraction II was accomplished by zone electrophoresis on Pevikon C870, as described by Müller-Eberhard (1960). The purification of α_1 -acid glycoprotein from fraction VI was achieved by column chromatography on Amberlite IRC-50 resin prepared as described by Hirs (1955). The resin (mesh 120-200) was equilibrated against 0.05 M-sodium citrate buffer, pH 5.20, and 10 mg. of protein was chromatographed on a 12 cm. x 1 cm. column. The elution was carried out with citrate buffer at the rate of 0.5-1.0 ml./hr. (Schmid, MacNair & Burgi, 1958).

The homogeneity of the purified proteins was checked by paper electrophoresis, starch-gel electrophoresis, agar-gel immunoelectrophoresis (Scheidtger, 1955) and/or starch-gel immunoelectrophoresis by a modification of the Poulik technique (1959).

In the last-named procedure, the starch-gel strip was sectioned in three layers and the top and bottom layers were stained with Amido Schwarz. The mid layer was placed in an appropriate tray which was filled with 1% agar. After

Scheme 1. Fractionation of rabbit plasma proteins. An explanation of the modifications is contained in the Methods section. A reference to the detailed procedure is in the same section. Fractions II and V were purified by zone electrophoresis on Pevikon and fraction VI by IRC-50 column chromatography. Details are given in the text.

cooling, troughs were cut in the agar parallel to the gel strip and filled with sheep anti-rabbit serum (Hyland Laboratories, Los Angeles, Calif., U.S.A.).

The protein concentration in the plasma and its fractions was determined by a biuret method (Mehl, 1945). The thiobarbituric acid procedure of Warren (1959) was selected to measure sialic acid, and the method described by Winzler (1955) served to determine protein-bound hexose. Hexosamine was measured by the method of Boas (1953). The fibrinogen content of fraction I and its supernatant was determined by the method of Jacobsson (1955) with the exception that the synerized clot was dissolved in NaOH-urea solution, as suggested by Blombäck & Blombäck (1956).

RESULTS

The average concentrations of total protein, sialic acid, hexosamine and protein-bound hexose in pooled rabbit plasma and in each fraction are presented in Tables 1 and 2. The separation of the α_1 - and α_2 -globulins and the resolution of the β_2 - and γ -globulins in fraction III were sometimes unsatisfactory; the relative concentration values of these proteins are therefore reported together in Table 1. However, good resolution of the globulin fractions was achieved when electrophoresis was carried out on freshly prepared specimens.

Table 1. *Fractionation of normal rabbit plasma proteins*

Electrophoresis was carried out in barbital-citrate buffer, pH 8.6 and *I* 0.1. The protein concentrations are average results of duplicate determinations (biuret method) from six complete fractionations. The results represent averages of 12 runs, except in fractions III and V, which are averages of six. Values are expressed as relative percentages. The average concentration of fibrinogen in fraction I was 70%.

	Concn. of protein (mg./100 ml. of plasma)	Albumin	α_1 -Globulin	α_2 -Globulin	β_1 -Globulin	β_2 -Globulin	γ -Globulin
Plasma	6070	54.4	11.6		11.9	10.9	11.2
Fraction I	517	10.0	+		8.1	65.5	16.3
Fraction II	915	1.2	7.0		11.3		80.5
Fraction III	1032	+	45.0		47.0		5.0
Fraction IV	140	26.0	46.0	18.0	8.3		1.7
Fraction V	3048	92.0	—	2.0	6.0		—
Fraction VI	35	20.0	62.0		17.0	1.0	—
Super-fraction II	20	37.0	+	—	35.6		27.4
Super-fraction V	27	69.0			23.0		6.0

Table 2. *Percentage distribution of protein-bound carbohydrate in the plasma fractions*

The results represent the averages of duplicate determinations on six complete plasma fractionations and refer to the percentage of constituent in each fraction.

	Total hexose (%)	Total hexosamine (%)	Total sialic acid (%)
Fraction I	6.7	7.5	8.0
Fraction II	15.3	22.0	8.0
Fraction III	38.7	33.0	45.0
Fraction IV	9.4	7.3	5.0
Fraction V	21.0	19.6	21.0
Fraction VI	8.9	6.0	8.0

Total serum hexose: 96 mg./100 ml.*

Total serum hexosamine: 75 mg./100 ml.*

Total serum sialic acid: 60 mg./100 ml.*

* Average results of duplicate determinations on 12 separate samples of normal rabbit sera.

The protein separation by starch-gel electrophoresis of the pooled rabbit plasma and its fractions is presented in Fig. 1. Each fraction displayed a complex but characteristic protein pattern, except fractions III and IV, which appeared similar by this technique but showed distinct differences on paper electrophoresis (Table 1). The haptoglobin distribution in the pooled plasma and in each fraction is shown in Fig. 2. The presence of these α_2 -globulins

Fig. 1. Stained starch-gel-electrophoretic patterns of pooled normal rabbit plasma and its fractions: (i) plasma 2; (ii) fraction VI; (iii) fraction V; (iv) fraction IV; (v) fraction III; (vi) fraction II; (vii) fraction I; (viii) plasma 1. Plasma 1 and fractions I, III and IV were analysed in one gel; plasma 2 and fractions II, V and VI were analysed in another. Both gels were prepared from the same batch and have a common standard. Electrophoresis was carried out for 11 hr. at 5° and pH 8.6 at 8 v/cm.; 4 mg. of protein was applied in each case.

Fig. 2. Starch-gel electrophoresis showing distribution of haptoglobins in plasma and fractions: (i) plasma; (ii) fraction VI; (iii) fraction V; (iv) fraction IV; (v) fraction III; (vi) fraction II; (vii) fraction I. The haptoglobin-haemoglobin complex was stained with dianisidine reagent. Fractions I and II were analysed separately. Electrophoresis was carried out for $14\frac{1}{2}$ hr. at 5° at 6.5 v/cm.

Fig. 3. Starch-gel electrophoresis of rabbit plasma and fraction III stained with *p*-phenylenediamine dihydrochloride: (i) fraction III; (ii) plasma. Visible oxidation of *p*-phenylenediamine dihydrochloride constitutes presumptive evidence that the protein is caeruloplasmin. The slower-migrating caeruloplasmin zone is faint. Electrophoresis was carried out for $14\frac{1}{2}$ hr. at 5° at 6.5 v/cm.

was demonstrated in all six fractions, but only one haptoglobin band was detected in fraction V and only traces of haptoglobins were seen in fraction VI.

The largest concentration of caeruloplasmin, according to the intensity and size of the coloured zone (*p*-phenylenediamine dihydrochloride and dianisidine reactions) was observed in fraction III, with smaller amounts in fractions II and IV. None was detected in fraction VI and only faint bands were seen in fractions I and V. Two caeruloplasmin bands were regularly observed in the pooled plasma and in fraction III with the *p*-phenylenediamine dihydrochloride reagent (Fig. 3). The first band in fraction III and in the pooled plasma was intense and broad and migrated ahead of the transferrin zone; the second band was faint and migrated behind the transferrin in the haptoglobin region.

Fig. 4. Vertical starch-gel electrophoresis of: (i) α_1 -acid glycoprotein; (ii) transferrin; (iii) γ -globulin; (iv) rabbit albumin. The third albumin component is very small and barely visible in the original. The transferrin shows several bands by Amido Schwarz staining after starch-gel electrophoresis in borate buffer. Electrophoresis was carried out for $14\frac{1}{2}$ hr. at 5° at 6.5 v/cm; 2 mg. of protein was applied in each case.

Only one transferrin band could be detected in the pooled plasma and in fraction V by radioautography. Similarly, transferrin eluted from the Pevikon block after electrophoresis displayed only one broad band by radioautography, but protein-staining of the other half of the gel strip revealed three small bands preceding and two following the main transferrin zone (Fig. 4). Paper electrophoresis and starch-gel immunoelectrophoresis showed one sharp zone and one precipitin arc respectively.

Albumin purified by Pevikon-block electrophoresis appeared homogeneous by paper electrophoresis, agar-gel immunoelectrophoresis and starch-gel immunoelectrophoresis, but revealed two components (and a trace of a third) on starch-gel electrophoresis (Fig. 4). γ -Globulin purified from fraction II showed one protein zone on paper electrophoresis and single precipitin arcs on starch-gel immunoelectrophoresis and agar-gel immunoelectrophoresis, but migrated both to the anode and to the cathode sides of the trough when analysed by starch-gel electrophoresis in borate buffer. The purified α_1 -acid glycoprotein was homogeneous on paper electrophoresis, starch-gel electrophoresis and starch-gel immunoelectrophoresis. The starch-gel electrophoretic mobility of rabbit α_1 -acid glycoprotein was very close to that of the human protein, but its concentration in rabbit plasma was one-third (12–16 mg./100 ml.) that in human plasma (30–40 mg./100 ml.).

DISCUSSION

The necessity of modifying the low-temperature low-salt ethanol procedure to obtain a satisfactory fractionation of rabbit plasma proteins was expected because of the difference in the composition of human and rabbit plasma proteins demonstrated by free electrophoresis (Moore, 1945; Deutsch & Goodloe, 1945) and by horizontal starch-gel electrophoresis (Engle & Woods, 1960). Comparison of the plasma-protein patterns of the two species by vertical starch-gel electrophoresis in borate buffer reveals more detailed differences: the absence of detectable amounts of pre-albumin I, the relative faintness of the α_1 -acid-glycoprotein zone in the rabbit plasma, the difference in mobility of transferrin in the two species, the difference in the post-albumin and haptoglobin patterns, the presence in rabbit plasma of a well-defined band migrating a short distance to the anode, which has been characterized recently as an α_1 -globulin, possibly a macroglobulin (Hemmings & Jones, 1963), and the difference in the pattern of migration of the γ -globulin in the two species (Fig. 5).

The fractionation scheme employed in the present study produced fractions with well-defined major electrophoretic components and, despite the

differences noted above, the results were comparable with those obtained in the separation of human plasma proteins by a similar procedure. Thus fraction I contains 90% of the fibrinogen, measured as clottable protein in the fraction and the supernatant. The γ -globulin is concentrated in fraction II. The β - and α -globulins are concentrated in fractions III and IV. Albumin constitutes over 90% of fraction V and nearly half of the total plasma protein. Fraction VI is small and contains mostly α_2 -globulins and α_1 -acid glycoprotein.

Attempts to separate rabbit plasma proteins by the unmodified application of the low-salt ethanol procedure successfully employed in the fractionation of human plasma failed to yield well-defined fractions IV, V and VI because part of the albumin was precipitated in fraction VI and could not be satisfactorily extracted from the precipitate containing fractions IV + V. This resulted in the distribution of albumin in all three fractions. In the present procedure, although albumin constitutes a significant percentage of fractions IV and VI, the total protein content of these two fractions is small so that only minute amounts of albumin are precipitated in fractions other than V. The extraction of fraction V from fractions IV + V was attempted at several pH values between 4.80 and 6.33, but the most satisfactory results were obtained at pH 4.90 and Zn^{2+} concentration 4 mM. This particular concentration of protons thus appears to be necessary to dissociate the Zn^{2+} -albumin complex and solubilize the albumin. The greater ability of Zn^{2+} to form a complex with and precipitate rabbit albumin as compared with human albumin may also account for the necessity of lowering the Zn^{2+} concentration in the precipitation of fractions IV + V to avoid significant contamination of fraction IV with albumin. The greater accessibility of imidazole groups, which are the binding sites of Zn^{2+} (Gurd & Goodman, 1952), could account for the observed behaviour of the rabbit plasma albumin in the fractionation procedure.

Several authors have reported the presence of more than one component when purified albumin is subjected to electrophoresis in starch gel. This has been attributed to polymerization (Saifer, Robin & Ventrice, 1961) and it has been observed after starch-gel electrophoresis in tris-citrate buffer of rabbit albumin prepared directly by zone electrophoresis on Pevikon (Bocci, 1962). This suggests that albumin polymerization is probably not dependent on the type of fractionation procedure employed. Albumin prepared by paper electrophoresis has been reported to be homogeneous on starch-gel electrophoresis by Poulik & Smithies (1958) and Saifer *et al.* (1961). However, Poulik, Zuelzer & Meyer (1961) reported the presence in several human sera of an albumin-like protein,

Fig. 5. Comparison of protein patterns in human and rabbit plasma by vertical starch-gel electrophoresis in borate buffer: (i) rabbit plasma; (ii) human plasma. Electrophoresis was carried out for 11 hr. at 5° at 8 v/cm.; 6 mg. of protein was applied in each case.

prepared by paper electrophoresis, which was resolved by two-dimensional starch-gel electrophoresis and became undetectable after treatment with 0.01 M 2-mercaptoethanol. Starch-gel immunoelectrophoresis of the purified rabbit albumin revealed only one continuous precipitin line deflected at the site of the dimer, thus indicating immunological relationship of the albumin and its dimer. The apparent absence of the albumin dimer in whole plasma and fraction V suggests that polymerization occurs during some phase of the purification procedure. Dialysis of fraction V through Sephadex and concentration by ultrafiltration instead of freeze-drying failed to prevent albumin polymerization.

Protein changes introduced during purification were also thought to account for the broad zone and the additional bands revealed by radioautography and staining with Amido Schwarz of the purified transferrin after starch-gel electrophoresis. However, this heterogeneity was still present when transferrin was prepared from fraction V with Rivanol (Boettcher, Kistler & Nitschmann, 1958) and subjected to electrophoresis in borate buffer, but was decreased when transferrin purified by zone electrophoresis on Pevikon was subjected to electrophoresis in tris-citrate buffer. The latter observation suggests that the borate buffer may be partially responsible for the heterogeneity observed. Heterogeneity of conalbumin detected by starch-gel electrophoresis in borate buffer has been reported by Parker & Bearn (1963). This heterogeneity was also diminished when electrophoresis was performed in tris-citrate buffer. Dialysis of the purified transferrin against 0.1 M-EDTA (disodium salt) produced a colourless but still heterogeneous protein; however, the pattern of resolution on starch-gel electrophoresis was altered slightly after this treatment. No instance of transferrin variants was detected in the pool of rabbit plasma by the technique of radioautography employed.

The detection of two caeruloplasmin bands in fraction III of pooled rabbit plasma is unlikely to be the result of an artifact, for the same bands are present in the unfractionated rabbit plasma, and multiple caeruloplasmin components have been reported in human serum (Morell & Scheinberg, 1960; McAlister *et al.* 1961).

The very low concentration of α_1 -acid glycoprotein in rabbit plasma explains the difficulty in detecting its presence on routine starch-gel electrophoresis. In view of the reported reactivity of this substance to disease (Nettelblad & Sundblad, 1962) its preparation and purification seemed particularly indicated in the context of this study.

The concentration of hexosamine and protein-bound hexose is lower in rabbit than in human plasma (Winzler, 1955), but the distribution of the

protein-bound carbohydrate is similar in both species and is maximally concentrated in fraction III (Table 2) (Lever *et al.* 1951).

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