Post-albumin, a Foetal-Specific Rat Plasma Protein

PURIFICATION, PHYSICOCHEMICAL AND IMMUNOLOGICAL STUDIES

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1. The foetal-specific post-albumin of rat plasma was purified by electrophoresis on a Pevecon block. 2. The sedimentation coefficient, diffusion coefficient and molecular weight were determined for this protein and found to be similar to those of adult rat plasma albumin. 3. Foetal post-albumin and adult albumin were compared immunologically and shown, with rabbit antisera, to share no antigenic determinants, suggesting different genetic control of the production of each. 4. It is suggested that the disappearance of post-albumin in the newborn animal may result from the disappearance of haemopoietic tissue from the rat liver with advancing age.

Differences in the plasma protein patterns of foetal and adult animals have been recognized for many years. Howe (1921) demonstrated differences in the albumin/globulin ratio of sera from newborn and adult animals. This difference was shown by Pedersen (1947) to be due to the presence of a foetal-specific α -globulin (fetuin) in the foetal calf. More recently, further differences in the plasma proteins of foetal and adult animals have been shown in a number of species. In the early human foetus (Andreoli & Robbins, 1962), the mouse foetus (Pantelouris & Hale, 1962) and the foetal rat (Wise, Ballard & Ezekiel, 1963), foetal-specific post-albumin components have been found in the plasma. In all cases the protein accounts for a large proportion of the total protein of foetal plasma.

Although the electrophoretic properties of rat post-albumin are similar to those of rat serum albumin, previous work on the labelling *in vivo* of the proteins with ^{14}C -labelled amino acids in the intact foetus suggested that independent synthesis occurs. Experiments *in vitro* demonstrated the synthesis of both post-albumin and albumin by foetal liver slices and that post-albumin was not synthesized by adult rat-liver slices or by placental slices (Wise & Oliver, 1966).

The present work describes the purification of post-albumin and its characterization by physicochemical and immunological criteria. Post-albumin

is similar to albumin in physical properties, but the serological reactions indicate that it is antigenically distinct from and probably unrelated to albumin.

MATERIALS AND METHODS

Foetal post-albumin

It was previously shown (Wise *et al.* 1963) that post-albumin can be successfully separated from foetal plasma by paper electrophoresis with the buffer of Aronsson & Gronwall, (1957). This buffer (0.5 M-tris-0.021 M-EDTA-0.075 M-boric acid, pH 8.9) was used for the preparation of post-albumin by electrophoresis in a Pevecon block (Pevecon C870; Superfosfat Fabriks, Stockholm, Sweden). A 3 ml. sample of pooled foetal plasma was dialysed against the buffer at 4°. The sample was coloured with bromophenol blue, which combines with native plasma albumin. Preliminary experiments with paper electrophoresis had shown that the dye does not bind to native post-albumin.

The Pevecon block was made up in a Perspex tray 50 cm. $\times$ 7 cm. $\times$ 0.75 cm., lined with Saran Wrap (Dow Chemical Corp., Midland, Mich., U.S.A.). A slot 5 cm. $\times$ 0.1 cm. was cut out of the block 10 cm. from the anode and the sample applied from a syringe. The edges of the slot were pressed together and the block was sealed with Saran Wrap. A potential difference of 700 V was applied for about 30 hr. at 4°.

On completion of the electrophoretic separation a strip of the block 1.0 cm. in width immediately behind the coloured albumin was removed and the liquid recovered by centrifugation. The Pevecon was washed twice with 0.15 M-NaCl and the combined washings were dialysed against distilled water before freeze-drying. In a typical preparation 9 mg. of protein was recovered.

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Physicochemical measurements

Sedimentation coefficients. These were determined by using the Spinco model E ultracentrifuge (Beckman Instruments, Palo Alto, Calif., U.S.A.) fitted with phase-plate schlieren optics and the RTIC system for temperature measurement and control.

Post-albumin was dissolved at various concentrations (0.4–1.5%) in 0.145 M-NaCl–0.02 M-tris adjusted to pH 7.45 with 2 N-HCl. Sedimentation-velocity runs were at 50740 rev./min. in the An-D rotor in 12 mm. aluminium centre-piece cells with 4° sector. The sedimentation coefficients were calculated by standard procedures (Schachman, 1957) and extrapolated to zero protein concentration. The protein shows little dependence of *S* value on concentration, and the sedimenting boundaries appeared symmetrical.

Diffusion coefficients. These were evaluated on a 0.4% soln. of post-albumin in the above buffer, by using the artificial-boundary cell in the An-D rotor. Speed was 13000 rev./min. and boundary spreading was followed for 72 min. after the establishment of the initial boundary.

The diffusion coefficient was evaluated from the equations:

$$\sigma^2 = \frac{1}{2\pi} \left(\frac{A}{H_{\max.}} \right)^2$$

$$\text{and } \frac{d\sigma^2}{dt} = 2D \text{ (Schachman, 1957)}$$

where *A* is the area under the schlieren curve of the diffusing boundary, *H*_{max.} the maximum ordinate of the curve, *t* the time and *D* the diffusion coefficient. The value was corrected to *D*_{20,w}. Molecular weight was calculated from the Svedberg equation (Svedberg, 1925) by using the values of *D*_{20,w} and *S*_{20,w} obtained on the 0.4% soln. of post-albumin. A partial specific volume of 0.73 was assumed in all the above calculations.

Serology

Antigens. Pooled rat foetal (18–22 days) and adult plasmas were obtained as described previously (Wise & Oliver, 1966). Rat plasma albumin was purified from adult plasma by repeated extraction with trichloroacetic acid-ethanol as described by Korner & Debro (1956), and foetal post-albumin from foetal plasma as described above.

Preparation of rabbit immune sera. (i) Antisera to rat plasma. Rat plasma (foetal or adult) was mixed with an equal volume of Freund's complete adjuvant (Commonwealth Serum Laboratories, Parkville, Victoria, Australia). Adult rabbits were given a subcutaneous injection of 2 ml. of the mixture and after 4 weeks given a second injection. Two further doses were then administered at weekly intervals. Commencing 2 weeks after the last injection, the rabbits were bled from the ear on alternate days for 1 week. The serum was separated from the blood clot and stored at –20°.

(ii) Antisera to purified rat plasma albumin. Rabbit antisera to rat albumin were prepared as described above by injection of a solution of rat albumin (1% in 0.15 M-NaCl) mixed in equal volume with Freund's adjuvant.

Immunoelectrophoresis. This was carried out, by the micro-modification of Scheidegger (1955), in agar gels made up to 1% in buffer of pH 8.6 (composition of the gel buffer: 2.9 mm-barbitone–17 mm-sodium barbitone–1.7 mm-calcium lactate, with 0.01% Merthiolate added as preservative;

composition of the buffer in electrode compartments: 7.5 mm-barbitone–42 mm-sodium barbitone–1 mm-calcium lactate). Electrophoresis was carried out at room temperature for 1 hr. with a gradient of 7–8 v/cm. The troughs were filled with the appropriate antisera and the slides incubated for 48 hr. at 20 ± 1° and then washed for 1 day in 0.15 M-NaCl to remove unprecipitated protein. After a rinse in distilled water, the slides were dried under lintless filter paper at 60°. The precipitin patterns were stained for 20 min. in Azocarmine B (0.05% solution in methanol-acetic acid–water, 5:1:4, by vol.) and washed in the solvent until the background was clear. Photography of the results was accomplished by direct projection on to hard Kodabromide paper.

RESULTS

The physicochemical parameters of post-albumin were determined as: *S*_{20,w}⁰ 4.43 × 10^{–13} sec. (4.43 s); *D*_{20,w} 6.25 × 10^{–7} cm.² sec.^{–1}; mol.wt. (Svedberg method) 64800.

Plate 1(a) shows the immunoelectrophoretic patterns of adult (top) and foetal (bottom) plasma developed with antiserum to foetal plasma. A single arc showing the characteristic albumin–anti-albumin reaction is visible in the adult pattern, but two such arcs appear in the foetal pattern. Starch-gel electrophoresis of plasma from foetal rats of this age similarly shows two components in high concentration migrating to the anode.

Plate 1(b) gives the pattern formed by purified rat plasma albumin (top) and foetal post-albumin (bottom) when allowed to react against antiserum to foetal plasma, with both antigens at 0.2% concentration. The contaminant visible in the foetal post-albumin pattern is also detectable in the rat plasma albumin when run at higher concentration. Although electrophoresis of post-albumin in starch gel suggests the presence of two components with almost equal mobility, the immunoelectrophoretic arc gives evidence neither of antigenic heterogeneity nor of bimodality even with extended electro-

EXPLANATION OF PLATE I

Micro-immunoelectrophoretograms of rat proteins allowed to react with rabbit antisera. (a) Whole plasma patterns of adult rat plasma (top well) and foetal plasma (bottom well) were developed with antiserum to foetal plasma (central trough). (b) Rat plasma albumin (top) and foetal post-albumin (bottom) were developed with antiserum to foetal plasma (trough). (c) As in (b), but with the antiserum trough shortened to allow diffusion of proteins around the end of the trough. (d) Rat plasma albumin (top) and foetal post-albumin (bottom) were developed with antiserum to rat plasma albumin. (e) Whole foetal plasma (top and bottom wells) and homogenate of 0.2 g. foetus (central well) were developed with antiserum to foetal plasma (top trough) and antiserum to adult plasma (bottom trough).

phoresis. Plate 1(c) shows the identical experiment, except that the antiserum trough was shortened so that its forward end was opposite to the centres of diffusion of albumin and post-albumin. Thus the proteins diffused around as well as towards the trough, and reacted with antibodies diffusing forward as well as laterally. The respective precipitin lines do not show blending, but cross in the reaction of non-identity.

In the experiment shown in Plate 1(d), the antiserum was to rat plasma albumin, and no reaction with post-albumin (bottom) took place. A faintly reacting component in the post-albumin sample (in the fast globulin region) is known not to be albumin, since the addition of albumin to the sample produces a separate arc.

In the experiment shown in Plate 1(e), three samples were run on a single slide and compared by means of two antisera. The top and bottom wells contained foetal plasma, and the middle well an extract in 0.15M-sodium chloride of a homogenate of an 0.2g. foetus (approx. 15 days gestation). The antiserum in the upper trough was anti-(foetal plasma) and in the bottom trough was anti-(adult plasma). Although the plasma from 18–22-day foetuses develops a strong albumin arc with both antisera, the extract of the younger foetus develops only a smear against this antiserum to adult plasma, which was shown to be albumin by the addition of albumin and the subsequent development of a single arc. It is noteworthy, too, that the antiserum to adult plasma was unable to detect post-albumin in either sample, even when the experiment was repeated with varying concentrations of the antiserum over an eightfold range. Taken with the evidence from other experiments, this result suggests that post-albumin is totally absent from the adult rat plasma; it is unlikely that the rabbit simply failed to produce antibodies against post-albumin in this case. However, the extract of the 0.2g. rat foetus gives a very strong reaction for post-albumin when allowed to react against anti-(foetal plasma) serum.

DISCUSSION

The major component of foetal rat plasma that is not found in either maternal or adult plasma has been purified and partially characterized. Ultracentrifugal analysis gave a single symmetrical boundary, but immunoelectrophoresis demonstrated the presence of a second weakly reacting antigen in the globulin region. Starch-gel electrophoresis gives suggestive evidence of microheterogeneity that was not, however, reflected by antigenic heterogeneity with the rabbit antisera used. Although foetal-specific proteins migrating directly behind albumin have been reported in a

number of species (Pedersen, 1947; Andreoli & Robbins, 1962; Pantelouris & Hale, 1962), only fetuin has previously been isolated and characterized. Fetuin isolated from calf serum has mol. wt. about 45 000 and a reported $S_{20,w}^0$ between 3 and 3.5s (Turner, 1963). On the other hand, rat post-albumin has $S_{20,w}^0$ about 4.4 and mol. wt. 65 000. These parameters are similar to those of rat plasma albumin (Charlwood, 1961). Fetuin migrates as an α -globulin whereas rat post-albumin is difficult to separate from albumin by conventional buffers for electrophoresis. For this reason its existence was not observed by Heim (1961) in a study of the serum proteins of the rat during development. On the basis of its electrophoretic mobility as well as its physicochemical properties, post-albumin is distinct from fetuin and can be classified as an albumin-like protein.

Although there are many points of similarity between rat plasma albumin and foetal post-albumin, the differences between these proteins are striking. Post-albumin does not bind bromophenol blue, a property that has been described in this paper as an aid in its separation from albumin. In addition, with the rabbit antisera used, no immunological cross-reactions were detected between post-albumin and albumin and this finding suggests that the two albumins possess different antigenic determinants. Therefore, in spite of the similarity in physical properties of post-albumin and albumin, their antigenic distinctiveness indicates that their tertiary structures, and hence their secondary and primary structures as well, must be considerably different.

In the most familiar example of a blood protein that exists in different forms in the foetus and adult, haemoglobin, the foetal form differs in 42 amino acid residues from the typical adult haemoglobin (Ingram, 1963). But these differences do not override the underlying and fundamental serological similarity of the proteins; there is considerable cross-reaction among these and other forms of haemoglobin when they are compared by serological methods (Schneider & Jones, 1965). This evidence and the gradual switch-over from haemoglobin F to A in the foetus argues strongly that some of the same genes are active in the production of both foetal and adult haemoglobins (Ingram, 1963). With foetal post-albumin and rat plasma albumin, however, the present serological experiments would seem to indicate that the proteins are unrelated, suggesting that different genes are active in the production of each.

Electrophoretic analysis of foetal plasma in starch gel (Wise *et al.* 1963) showed that the concentration ratio of post-albumin to albumin is greatest in early foetuses and decreases with advancing age. In the current experiments, albumin

was only barely detectable by the serological technique in extracts of 0.2g. rat foetus, whereas post-albumin was present in high concentration. Therefore it seems likely that post-albumin is one of the first plasma proteins to be produced by the foetus. Wise & Oliver (1966) have shown that both albumin and post-albumin are synthesized by the foetal liver, but it is noteworthy that the decrease in concentration of post-albumin that occurs in the developing foetus is roughly parallel to the decrease in the amount of haemopoietic tissue that occurs during perinatal liver maturation (Oliver, Blumer & Witham, 1963). It is possible that the cellular site of synthesis of post-albumin is different from that of albumin, and that the haemopoietic tissue in the embryonic liver may be implicated in the synthesis of this protein.

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