Precursor-Product Relationship between Intrahepatic Albumin and Plasma Albumin

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Rats were injected with [^3H]leucine, and at various times thereafter labelled albumin was isolated by electrophoresis from their livers and blood plasma. The specific radioactivity of each protein was determined by spectrophotometry and liquid-scintillation spectrometry. Intrahepatic albumin was shown to be identical with plasma albumin by its electrophoretic mobility and antigenicity. It was found that intrahepatic albumin was the direct precursor of plasma albumin. Comparison of their specific radioactivities showed that intrahepatic albumin attained a higher specific radioactivity before plasma albumin. When plasma albumin reached its maximum specific radioactivity, that of intrahepatic albumin had decreased to a similar value. Thereafter, the specific radioactivity of intrahepatic albumin remained lower than that of plasma albumin.

It is well established that normally-occurring plasma proteins, except the γ -globulins, are synthesized by the liver. This unique function was proposed by Madden & Whipple (1940), who observed that dogs whose livers were deprived of portal blood were unable to form significant amounts of plasma proteins. Miller & Bale (1954) showed that the isolated perfused rat liver incorporated labelled amino acids into plasma proteins of the perfusion medium. Campbell & Stone (1957) found that liver slices synthesized labelled albumin when incubated with [^{14}C]glycine and Wise & Oliver (1966) injected rat embryos with ^{14}C -labelled algal-protein hydrolysate and observed incorporation of the label into foetal plasma proteins.

In the above studies conclusions were drawn from observations on proteins in the blood, but not from proteins in the liver. However, Peters (1962) followed ^{14}C -labelled albumin through the various intracellular compartments within the liver. Intrahepatic albumin was identified by immunoprecipitation, and the results were expressed as a percentage of the injected dose. Stating the results in this manner enabled comparison to be made of temporal values within compartments but not between compartments, e.g. between the soluble cytoplasmic fraction and the blood plasma. Such a comparison can be made only if changes in the specific radioactivity (disintegrations/min./ μg . of albumin) are followed with time. This comparison of specific radioactivities with time can be accomplished by using the precursor-product relations outlined by

Zilversmit, Entenman & Fishler (1943). In essence, this relationship states that if a substance A is a direct precursor of substance B, a graph of the kinetics of their specific radioactivities should reveal that the specific radioactivity of substance A attains its highest value before that of substance B, and the descending slope of the specific radioactivity of substance A should cross at the highest value of that of substance B, thereafter remaining below it in value. In the present work, the specific radioactivity of intrahepatic albumin was compared with the specific radioactivity of plasma albumin at various times after injection of [^3H]leucine into rats.

MATERIALS AND METHODS

[^3H]Leucine injection. Male Sprague-Dawley rats (Simonsen Farms, Gilroy, Calif., U.S.A.), weighing 240–300 g. were injected via the external jugular vein with $10\mu\text{C}$ of [$\text{G-}^3\text{H}$]leucine (specific radioactivity 3.2C/m-mole/g . body wt. The labelled leucine was purchased from International Chemical and Nuclear Corp., City of Industry, Calif., U.S.A. The purity of the [^3H]leucine was checked by paper chromatography in two solvent systems. More than 99% of the radioactivity had an R_F identical with that of unlabelled leucine.

The rats were killed at 0.25, 0.5, 0.75, 1, 2, 4, 6, 8 and 12 hr. after injection of the [^3H]leucine. The rats were anaesthetized by an intraperitoneal injection of Nembutal (0.3 mg./g. body wt.).

Preparation of proteins. Crude concentrated soluble liver proteins were obtained by a method described previously (LeBouton, 1967).

Blood was withdrawn from the inferior vena cava into a syringe pre-wetted with 3.1% (w/v) sodium citrate and then transferred to a centrifuge tube containing 0.1 ml. of 3.1% (w/v) sodium citrate/ml. of whole blood. After centrifugation of the citrated blood the clear straw-coloured plasma was recovered and stored at 4°. To purify albumin partially, plasma was fractionated at 4° with a saturated $(\text{NH}_4)_2\text{SO}_4$ solution according to Pennel (1960). The sediment produced by 50%-saturated $(\text{NH}_4)_2\text{SO}_4$ was washed once with 50%-saturated $(\text{NH}_4)_2\text{SO}_4$ and then dissolved in the original plasma volume of veronal buffer, pH 8.6, 1.0-0.5 (Bailey, 1962). Although the 50%-saturated $(\text{NH}_4)_2\text{SO}_4$ fraction of plasma contains mainly globulins (Pennel, 1960), it has also been shown to contain a significant amount of albumin (Dixon & Webb, 1961; Roche & Derrien, 1946).

Electrophoresis of proteins. Liver proteins and proteins in the 50%-saturated $(\text{NH}_4)_2\text{SO}_4$ fraction of plasma were separated on cellulose acetate (Cellogel; Colab Laboratories, Chicago Heights, Ill., U.S.A.) in veronal buffer, pH 8.6, 1.0-0.5 and then fixed and stained with fast green dye (LeBouton, 1967). Cellogel is a pre-wetted gelatinized cellulose acetate that does not reveal any detectable shrinkage during the fixation and staining process. A potential of 20 v/cm. was applied to the 3.5 cm. $\times$ 10 cm. cellulose acetate strips for 3 hr. About 10–15 μ l. of each sample was placed 3 cm. from the cathode of a model 51170 electrophoresis tank obtained from Gelman Instrument Co., Ann Arbor, Mich., U.S.A. The migration of each protein in millimetres was measured from the middle of the origin to the middle of the protein band, and migration rates were calculated from the formula given by Wunderly (1961).

Measurement of protein. The plasma albumin band and the liver protein band having a migration rate closest to that of plasma albumin were cut off the cellulose acetate and dissolved in 1 ml. of 10% (v/v) acetic acid in acetone. Only the middle 1 mm. of the large plasma albumin band was removed, whereas the smaller intrahepatic albumin band was entirely removed. After each band had dissolved, the light-green fluid was decanted into a 10 mm. $\times$ 75 mm. cuvette, which was immediately stoppered. All cuvettes were matched to within $\pm 0.6\%$ accuracy at 42% transmittance. The extinction of each dissolved band was determined in a Coleman Junior spectrophotometer at 615 m μ against a blank of an equal volume of 10% (v/v) acetic acid in acetone containing an unstained portion of cellulose acetate. The amount of protein in each dissolved protein-dye complex was estimated by comparing its extinction with a standard concentration-extinction calibration graph prepared with known amounts of albumin.

Measurement of radioactivity. The contents of each cuvette were decanted into 22 ml. glass vials (Wheaton Glass Co., Millville, N.J., U.S.A.). Cuvettes were washed once with 10% (v/v) acetic acid in acetone and the wash was added to the vial. The contents of the vials were evaporated, leaving the cellulose acetate and protein-dye complex as a thin residue on the bottom of the vial.

Nuclear-Chicago solubilizer (0.5 ml.) (Nuclear-Chicago Corp., Des Plaines, Ill., U.S.A.) was added to each vial (Hansen & Bush, 1967). The vials were capped and autoclaved at 115° for 1 hr. to decolorize the dye and solubilize the protein. After cooling, the clear solution was acidified with 0.3 ml. of conc. HCl to decrease initial phosphorescence (Steinberg, Vaughan, Anfinsen, Gorry & Logan, 1958). Then 15 ml. of scintillation solvent consisting of 5 g. of

2,5-diphenyloxazole and 0.6 g. of 1,4-bis-(5-phenyloxazol-2-yl)benzene/l. of toluene (A.R.) was added to each vial. The vials were placed in the sample conveyor of a mark I liquid-scintillation spectrometer (Nuclear-Chicago Corp.), where they were left at 0° overnight before being counted.

All samples were counted to a pre-set value of 10000 counts and background was automatically subtracted. The counting channels were operated at balance point and discriminators were set from 0.5 v to 9.9 v to include the entire energy spectrum of tritium. Counting efficiency was determined by the channels-ratio method (Bush, 1963). The majority of samples were counted at near 30% efficiency.

Immunological identification of albumin. Duplicate samples of liver supernatant fluid, whole plasma and the 50%-saturated $(\text{NH}_4)_2\text{SO}_4$ fraction of plasma were separated by electrophoresis on cellulose acetate as described above. One strip from each duplicate sample was fixed and stained, while the other strip was kept frozen on solid CO_2 . The unstained strips were removed from the solid CO_2 and aligned on a glass plate so that the origin of each was opposite that of its stained duplicate. The area on the unstained plasma strip corresponding to the albumin band on the stained plasma strip was cut out, rolled up and placed in one of the peripheral wells of an immunodiffusion plate (Mann Research Laboratories, New York, N.Y., U.S.A.). Unstained areas from the liver supernatant fluid and the 50%-saturated $(\text{NH}_4)_2\text{SO}_4$ fraction of plasma were treated similarly. Each peripheral well was filled with 0.1 ml. of 0.15 M-NaCl, and the centre well contained 0.1 ml. of a rabbit antiserum to rat serum (Mann Research Laboratories). The plate was developed at room temperature for 2 days.

As a check on the efficacy of protein removal the remains of each unstained strip were stained in the normal manner. In every case the desired protein band was shown to have been removed.

RESULTS

The average amounts of plasma albumin and intrahepatic albumin measured by spectrophotometry of the bound dye were 10.8 μ g. and 5.9 μ g. respectively. These values were within the linear part of the standard concentration-extinction plot that conformed to Beer's law.

The results of a typical immunodiffusion plate are shown in Fig. 1. Two precipitation lines were formed by the interaction of the antiserum and albumin from whole plasma; the smaller of these lines did not exhibit a reaction of identity with any of the other lines on the plate. However, the larger of these two lines exhibited a reaction of identity with intrahepatic albumin from the liver supernatant fluid; this line was continuous with the precipitation line from albumin in the 50%-saturated ammonium sulphate fraction of plasma.

Fig. 2 shows the incorporation of [^3H]leucine into plasma albumin and intrahepatic albumin as a function of time. Intrahepatic albumin attained a maximum specific radioactivity of 34 disintegrations/min./ μ g. 1.5 hr. before plasma albumin

Fig. 1. Diagram of immunodiffusion patterns of plasma albumin and intrahepatic albumin resulting from interaction with a rabbit antiserum to rat serum. Proteins were separated by electrophoresis on cellulose acetate and areas of cellulose acetate containing the desired unfixed proteins were cut out, rolled up and placed in peripheral wells with 0.1 ml. of 0.15M-NaCl. The antiserum was placed in the centre well. PA, albumin from whole plasma; LA, intrahepatic albumin from liver supernatant fluid; PAF, albumin from the 50%-saturated ammonium sulphate fraction of plasma.

equal to that of plasma albumin. Thereafter, the specific radioactivity of intrahepatic albumin remained below that of plasma albumin. The half-life of intrahepatic albumin is estimated from Fig. 2 to be 70–80 min.

From 18 determinations, plasma albumin migrated an average of 4.8 cm. in 3 hr., which is a rate of 2.2×10^{-5} cm./sec./ $\sqrt{v}$ /cm. Intrahepatic albumin migrated an average of 5.1 cm. in 3 hr., the rate of migration being 2.4×10^{-5} cm./sec./ $\sqrt{v}$ /cm.

DISCUSSION

Intrahepatic albumin has previously been identified by immunoelectrophoresis (Hase & Mahin, 1965), immunoprecipitation (Peters, 1962), amino acid composition (Müller, 1964) and the fluorescent-antibody technique (Hamashima, Harter & Coons, 1964). Since in the present study only intrahepatic albumin was of interest among many proteins on the cellulose acetate strip, it was easier to separate the albumin from the other proteins and then identify it by immunodiffusion. This procedure precludes the interference from other proteins encountered with immunoelectrophoresis, and obviates the rigorous preparations required with the fluorescent-antibody technique.

Peters (1962) found that intrahepatic albumin attained peak labelling about 30 min. after injection of [14 C]leucine into a rat. This is in complete agreement with the time of highest specific radioactivity of intrahepatic albumin seen in the present study (Fig. 2). Also, the half-life of intrahepatic albumin in the present work (70 to 80 min.) is quite close to the value of 80 min. that can be estimated from Fig. 2 of Peters (1962).

The initial increase in specific radioactivity of plasma albumin 15 min. after injection cannot be explained (Fig. 2). It was at first thought that it could be due to binding of free [3 H]leucine by the albumin. However, binding of amino acids like leucine that are neutral at the pH of blood does not occur (Hunter & Commerford, 1955; Lietze, Haurowitz & Turner, 1958).

Since the initial work by Zilversmit *et al.* (1943) the concept of the precursor-product relationship has not been extensively utilized, although the desirability of such data has been discussed by Funabiki & Kondutsu (1965). Inherent difficulties in this approach, as in general protein chemistry, are the problems of isolation and identification. By combining electrophoresis (isolation) with immunodiffusion (identification), the relationship between precursor and product can be observed. Thus, as seen in Fig. 2, intrahepatic albumin is indeed the direct precursor of plasma albumin.

The migration values (in units of 10^{-5} cm./sec./ $\sqrt{v}$ /cm.) of intrahepatic albumin (2.4) and plasma

Fig. 2. Specific radioactivities of intrahepatic albumin (●) and plasma albumin (○) as functions of time after injection of [3 H]leucine into rats. Albumins from the liver supernatant fluid and from the 50%-saturated ammonium sulphate fraction of plasma were isolated by electrophoresis on cellulose acetate. The proteins were fixed and then stained with fast green dye. Stained albumin bands were cut from the cellulose acetate and their protein content was determined by spectrophotometry of the bound dye. Radioactivity of protein in the bands was measured by liquid-scintillation spectrometry. Each point is the average of two determinations.

reached its maximum specific radioactivity. After 1.5–2 hr., when plasma albumin reached its highest value of 14 disintegrations/min./μg., the specific radioactivity of intrahepatic albumin was almost

albumin (2.2) are much lower than the published value for rat plasma albumin (6.1) in paper electrophoresis (Spector, 1956). This is not surprising since, as pointed out by Kunkel & Tiselius (1951), the path taken by a protein particle under electrophoresis is not the shortest one, but instead is much longer, depending on the tortuosity of the electrophoretic medium. Since cellulose acetate is microporous (Kohn, 1960), the path taken by a protein particle migrating through it would probably be longer than the path taken by the same protein through relatively coarse-pored filter paper, and hence its apparent migration rate uncorrected for this tortuosity factor would appear smaller.

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