Biosynthesis of Serum Albumin in Rat Liver

EVIDENCE FOR THE EXISTENCE OF 'PROALBUMIN'

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1. A protein(s) of rat liver (precipitated from soluble extracts of the microsomal fraction by anti-albumin) yields albumin after limited hydrolysis by trypsin. 2. Evidence that the product of limited tryptic hydrolysis is albumin, is based upon ion-exchange chromatography, electrofocusing and peptide 'mapping'. 3. The albumin 'precursor' is recognized by anti-albumin and is apparently not distinguished from albumin by anti-albumin. 4. A small peptide is liberated from the presumptive albumin precursor during limited tryptic hydrolysis. This peptide is labelled by arginine, but not by leucine, lysine or methionine. 5. These results support our previous suggestion based on kinetic evidence that the albumin-like protein(s), in the anti-albumin precipitate from rat liver, is an albumin precursor.

In previous papers (Judah & Nicholls, 1971*b*; Geller *et al.*, 1972) we suggested that rat serum albumin was synthesized by way of a polypeptide precursor. Evidence for this conclusion was derived from work with liver slices as well as with whole rats. In the present paper we give evidence that the protein(s) closely resembling rat serum albumin, which we suggested had the properties of a precursor, may indeed be converted into serum albumin by tryptic hydrolysis.

Materials and Methods

All the methods and materials are the same as those employed previously (Judah & Nicholls, 1971*a,b*; Geller *et al.*, 1972) with the following exceptions.

Purification of rat serum albumin

Crude serum albumin was prepared by the method of Debro *et al.* (1957). A portion (400 mg) of this material was subjected to chromatography on Sephadex G-200 in a column (85 cm $\times$ 2.5 cm) with 1 M-NaCl-100 mM-Tris-HCl, pH 7.8, as eluent. The flow rate was 30 ml/h. About 300 mg of albumin was recovered after fractions at the extremes of the protein band had been discarded. The product is free of α -globulin, as shown by electrophoresis on cellulose acetate, and gives a single precipitin line on immunoelectrophoresis. It appears as a pure product on electrofocusing on polyacrylamide gel (kindly done by Dr. A. H. Gordon, National Institute for Medical Research, London NW7 1AA, U.K.) and on disc electrophoresis on polyacrylamide gel.

Radioactive materials

Labelled amino acids. L-[1- 14 C]Leucine, L-[4,5- 3 H]-leucine, L-[U- 14 C]arginine, L-[U- 14 C]lysine and L-[G- 3 H]methionine were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. The labelled amino acids were given to rats (approx. 150 g body wt.) by intravenous injection in a tail vein. The doses were 40 μ Ci of 14 C-labelled amino acid or 200 μ Ci of 3 H-labelled amino acid unless otherwise specified.

Determination of radioactivity. This was done by liquid-scintillation spectrometry, as described by Judah & Nicholls (1971*a,b*).

Preparation of labelled albumin from serum. The method described above for purification of serum albumin was satisfactory, but since smaller amounts of protein were handled, a column of Sephadex G-200 of 30 cm $\times$ 1 cm sufficed. The animals were killed 60 min after administration of labelled amino acid (see above).

Preparation of labelled 'precursor'. For most purposes, an anti-albumin precipitate was obtained from deoxycholate extracts of a rat liver microsomal fraction (Judah & Nicholls, 1971*a*). No carrier albumin was added. The soluble antigens were separated (Judah & Nicholls, 1971*a*) to yield approx. 0.7 mg of protein (as measured by E_{280}) from the microsomal fraction of one liver (approx. 7 g wet wt.). The animals were killed 10 min after the injection of labelled amino acid and the antigens contained approx. 300 000 d.p.m. of 14 C when the label was 40 μ Ci of [14 C]leucine. About 90% of the radioactivity and about 30% of the protein so collected was in the form of precursor, the remainder being in the form of serum albumin. For most of the experiments, this

crude preparation was used as source of radioactive precursor since (see below) carrier albumin was added to the system and the small amount of radioactive albumin in the 'precursor' preparation was of no concern.

'Precursor' labelled with [^{14}C]arginine or [^{14}C]lysine was sometimes contaminated by low-molecular-weight radioactive impurities. These were removed by passage through Sephadex G-25 (see below).

Preparation of radioactive 'precursor' free of serum albumin. This was done by chromatography on DEAE-cellulose. An antibody precipitate was made from five rat livers (one of which was labelled by intravenous injection of $40\mu\text{Ci}$ of L-[^{14}C]leucine). The yield after separation of the soluble antigens was 3.8mg of protein and 300000 d.p.m. of ^{14}C . This was applied to a DEAE-cellulose column ($40\text{cm} \times 1\text{cm}$) in 2ml of 150mM-Tris-HCl, pH 7.8, and eluted by a stepwise elution with 40ml of 150mM-Tris-HCl, pH 7.8, followed by 20ml of 175mM-Tris-HCl, pH 7.8, and then 40ml of 200mM-Tris-HCl, pH 7.8. A flow rate of 15ml/h was used and 2ml fractions were collected. The 'precursor' emerged in a sharp band between fractions 35 and 40. The five peak tubes were pooled to yield 0.5mg of protein of sp. radioactivity 150000 d.p.m./mg. Serum albumin emerged between tubes 44 and 49; 1.2mg of protein of sp. radioactivity 21000 d.p.m./mg was recovered.

Chromatographic procedures

Sephadex chromatography. This was done with Sephadex G-10 or G-25 in columns of $40\text{cm} \times 1\text{cm}$. Sephadex G-200 was used in a column of $33\text{cm} \times 2.5\text{cm}$. The eluent was 10mM-Tris-HCl, pH 7.8, at a flow rate of 15ml/h in all cases, unless otherwise specified.

Chromatography on DEAE-cellulose. DE 52 (obtained from Whatman Biochemicals Ltd., Maidstone, Kent, U.K.) was used in columns ($40\text{cm} \times 1\text{cm}$) with linear gradients of 100ml of each of 150mM-Tris-HCl, pH 7.8, and 250mM-Tris-HCl, pH 7.8. The flow rates were 15ml/h. Fractions of 2ml were collected. The pH was measured at 21°C . Exceptions to these conditions are noted in the relevant places.

Electrofocusing in pH gradients in the presence of 6M-urea. This was done in linear sucrose gradients of pH 5-7 (Haglund, 1971) by using a LKB 8100 column (LKB, Bromma, Sweden). The urea solution, 9M, freshly made with Aristar grade urea (BDH Ltd., Poole, Dorset, U.K.) was passed through a mixed-bed ion-exchanger. This solution was then used to make up the sucrose gradient and heavy electrode solution to yield a final concentration of 6M-urea.

If a relatively large volume of sample had to be used, this was made to a final concentration of 6M-urea and replaced all or part of the 'light gradient' solution.

Protein determinations. These were done by the method of Lowry *et al.* (1951) or by reading absorbance at 280nm. Rat serum albumin was used as standard.

Immunological procedures

Double-antibody method for precipitation of 'precursor'. Anti-(rat albumin) antibody raised in rabbits (prepared for us by Burroughs Wellcome Research Laboratories, Beckenham, Kent, U.K., against purified rat serum albumin supplied by us) was allowed to react for approx. 16h at 2°C with an excess of anti-(rabbit γ -globulin) raised in sheep (obtained from Burroughs Wellcome Research Laboratories). This complex was titrated with ^{14}C -labelled serum albumin to determine how much was needed to bind 9-10 μg of antigen. This amount of the complex was then allowed to react with ^{14}C -labelled 'precursor' (separated from serum albumin as described above) or with varying amounts of unlabelled serum albumin followed by precursor. The mixture was kept overnight at approx. 2°C . It was then centrifuged (6000g_{av} for 10min), the solid washed twice in ice-cold 150mM-NaCl and the radioactivity determined.

Titration of 'precursor' and albumin with antibody. A constant mixture of ^{14}C -labelled precursor (20 μg of protein, 13500 d.p.m.) and 150 μg of unlabelled serum albumin was exposed to a range of anti-albumin concentrations from a low value sufficient to precipitate about 15 μg of albumin to a high value sufficient to precipitate 175 μg of albumin. The precipitate was washed three times in ice-cold 150mM-NaCl, the antigens were released (Judah & Nicholls, 1971a) and the specific radioactivity of the precipitated proteins was determined.

Proteolytic enzymes

Trypsin. Crystalline trypsin (for analysis), crystalline chymotrypsin (for analysis) and crystalline papain (for analysis) were obtained from Boehringer Corp., London W.5, U.K. The trypsin was standardized by its hydrolysis of benzoylarginine ethyl ester (Schwert & Takenaka, 1955) and found to possess the activity specified by the suppliers (10000 units/mg of protein as defined by Schwert & Takenaka, 1955).

Systems for limited proteolysis of 'precursor'. Trypsin (1 $\mu\text{g}/\text{ml}$) was incubated for 60min at 21°C with unlabelled carrier albumin (5.0-15mg), 'precursor' solution (containing about 70-150 μg of antibody-precipitable protein), 50mM-Tris-HCl, pH 7.8, and 50 μM - CaCl_2 , final volume 1ml. The reaction was stopped by addition of 10 μg of soyabean trypsin inhibitor [Sigma (London) Chemical Co., London S.W.6, U.K.] and the reaction mixture was passed at once on to DEAE-cellulose of electrofocusing columns for identification of product. Zero-time

controls were done by addition of soyabean trypsin inhibitor before incubation. Chymotrypsin (2.5 and 50 $\mu\text{g/ml}$) was used as described for trypsin. Papain (10–50 $\mu\text{g/ml}$) was tested in a system containing carrier albumin and 'precursor' (as specified above), 1.0 mM-2-mercaptoethanol, 0.2 mM-EDTA and 50 mM-potassium phosphate buffer, pH 6.0, final volume 1 ml. The temperature of incubation was 21°C and duration 60 min. The reaction was stopped by addition of trichloroacetic acid (5%, w/v, final concn.), the precipitate dissolved in ethanol and the proteins were precipitated therefrom by addition of 2 vol. of diethyl ether. The protein was then dissolved in 2 ml of 150 mM-Tris-HCl, pH 7.8, for chromatography on DEAE-cellulose as specified above.

Peptide 'mapping' of albumin and 'precursor'

This was done by the method of Huehns (1968). The electrophoresis buffer was pyridine-acetic acid-water (10:0.4:90, by vol.). The chromatography solvent was 3-methylbutan-1-ol-pyridine-water (35:35:27, by vol.). Twice-crystallized trypsin (Worthington Biochemicals Corp., Freehold, N.J., U.S.A.) was used for hydrolysis. About 400–500 μg of enzyme was added to the 20–30 mg of albumin used for peptide 'mapping' in a final volume of 1 ml.

Leucine-labelled 'precursor' and albumin. Serum albumin marker was labelled with L-[4,5- ^3H]leucine (1 mCi) given by way of the tail vein to a rat, which was killed 60 min later. The serum was collected and albumin was purified as described above. 'Precursor' was labelled with an intravenous dose of 100 μCi of L-[1- ^{14}C]leucine, and the protein separated from the liver 10 min after injection of the label as described above.

Two types of peptide 'mapping' were done. (1) With a mixture of 16.5 mg of ^3H -labelled albumin (2.8×10^6 d.p.m.) and ^{14}C -labelled 'precursor' (0.6 mg of protein, 225 000 d.p.m.). (2) With a mixture of ^3H -labelled albumin and ^{14}C -labelled precursor after limited tryptic hydrolysis. For this experiment ^{14}C -labelled 'precursor' (1.2 mg of protein, 670 000 d.p.m.) was mixed with ^3H -labelled albumin (75 mg of protein, 2.8×10^6 d.p.m.) and trypsin (1 $\mu\text{g/ml}$), 40 μM - CaCl_2 , 40 mM-Tris-HCl, pH 7.9, final volume 7.4 ml. The mixture was incubated for 50 min at 21°C. The reaction mixture was then applied to a Sephadex G-200 column and eluted as described above. The contents of five peak tubes (15 ml total volume) were collected and concentrated to 1 ml. The yield was 30 mg of protein (containing 270 000 d.p.m. of ^{14}C and 1.13×10^6 d.p.m. of ^3H). This solution was used for the peptide 'map'.

The stained peptide 'map' was examined in two ways. (1) It was applied to X-ray film (Kodak RP14 ester base) in a cassette (Kodak Ltd., Hemel Hempstead, Herts., U.K.) (35 cm $\times$ 43 cm), equipped with

intensifying screens and left for 8 weeks to make a radioautograph. (2) The spots were carefully cut out, numbered and counted for radioactivity in the scintillation system described by Judah & Nicholls (1971a,b). The $^{14}\text{C}/^3\text{H}$ ratios were calculated after background subtraction and allowance for 'break-through' of ^{14}C into the ^3H channel (approx. 10%).

This method was in turn controlled by mixing ^{14}C -labelled albumin and ^3H -labelled albumin, preparing a peptide 'map' as described above and obtaining the $^{14}\text{C}/^3\text{H}$ ratios as described above.

[^{14}C]Arginine- and [^{14}C]lysine-labelled 'precursor' and albumin. For these experiments 'precursor' was obtained from two rats, one given 50 μCi of L-[U- ^{14}C]arginine and the other given 50 μCi of L-[U- ^{14}C]lysine. Crude 'precursor' preparations were obtained as described above and mixed, yielding 1.3 mg of total protein and 288 000 d.p.m. of ^{14}C . To this was added 20 mg of carrier albumin and the mixture was used to obtain a peptide 'map'.

Serum albumin labelled with [^{14}C]arginine and [^{14}C]lysine was obtained in exactly the same way as described above, by using two rats each dosed with 50 μCi of L-[U- ^{14}C]arginine or 50 μCi of L-[U- ^{14}C]lysine. The crude albumin preparations were mixed (17.2 mg of [^{14}C]arginine-labelled albumin, sp. radioactivity 5400 d.p.m./mg) and passed through a Sephadex G-200 column. The peak tubes (22 mg of albumin, sp. radioactivity 12700 d.p.m./mg) were concentrated and used for peptide 'mapping'. The stained peptide 'maps' were used for radioautography as described above.

Results and Discussion

Effect of enzymes on 'precursor'

Effect of trypsin. Fig. 1(a) shows a typical chromatogram of 'precursor' labelled with L-[1- ^{14}C]leucine run on DEAE-cellulose before trypsin treatment. This is fully in accord with previous findings (Judah & Nicholls, 1971a). Fig. 1(b) shows the effect of incubation with trypsin in the system described in the Materials and Methods section. If soyabean trypsin inhibitor is added before trypsin, the pattern remains identical with that shown in Fig. 1(a).

The relationships of 'precursor' to albumin are the same when the 'precursor' was labelled with L-valine, L-lysine, L-arginine or L-methionine. When the 'precursor' was labelled with a mixture of [^3H]lysine and [^{14}C]leucine, the labels could not be separated from each other.

These experiments were repeated; 27 DEAE-cellulose chromatograms were run on 19 separate 'precursor' preparations after tryptic digestion, and the zero-time (control) preparations were run 19 times.

Effect of chymotrypsin and papain. Chymotrypsin (at 2.5 and 50 $\mu\text{g/ml}$) and papain (at 10 $\mu\text{g/ml}$) failed

Fig. 1. Chromatography on DEAE-cellulose of 'proalbumin' before (a) and after (b) trypsin treatment

The conditions for limited hydrolysis were as described in the Materials and Methods section. Trypsin inhibitor was added (a) at zero time or (b) after 60 min incubation. The sample contained 15 mg of carrier rat serum albumin, together with 'proalbumin' [$37\mu\text{g}$ of protein (a) or $74\mu\text{g}$ of protein (b)] of specific radioactivity 415000 d.p.m./mg of protein, labelled with L-[1- ^{14}C]leucine as described in the Materials and Methods section. DEAE-cellulose chromatography was done with a linear salt gradient as described in the Materials and Methods section. $\circ$, E_{280} ; $\bullet$, ^{14}C radioactivity.

to influence the pattern shown in Figs. 1(a) and 1(b). Papain (at $50\mu\text{g}/\text{ml}$) gave a modest shift of radioactivity into albumin, but this relative failure of papain might be due to the presence of carrier albumin, which could compete for papain.

Products of trypsin attack on 'precursor'. When [^{14}C]arginine-labelled 'precursor' is exposed to trypsin, and then run on DEAE-cellulose, a fraction of the radioactivity appears near the front of the chromatogram (peak I, Fig. 2). This radioactive material, when chromatographed on Sephadex G-10, runs near the front of the elution pattern. It is fully retained on Sephadex G-25, and is thought therefore to have an apparent molecular weight of about 600. It is not antibody-precipitable and can easily be separated from free [^{14}C]arginine on Sephadex G-10 columns, which retain the free amino acid fully. Peaks II and III in the chromatogram were antibody-precipitable and could not be separated from the starting 'precursor' by chromatography on Sephadex G-200. They were presumed to be unchanged (or partially changed) precursor. Peak IV

coincided with the E_{280} peak of marker serum albumin.

This experiment was repeated by using a different sequence of operations. A preparation of [^{14}C]arginine-labelled precursor (0.62 mg of protein, 100000 d.p.m.) was passed through Sephadex G-25 to free it of possible low-molecular-weight contaminants. A portion was treated with trypsin and passed through Sephadex G-25 again. Approx. 10% of the label appeared after approx. 30 ml had passed through the column ($V_t = 30\text{ ml}$). The remainder of the protein and radioactivity appeared at the front of the elution and was subjected to DEAE-cellulose chromatography, which showed the transformation seen in Fig. 1(b). Precursor not treated with trypsin gave no sign of the appearance of peptide in a control of this experiment, and DEAE-cellulose chromatography of the proteins at the front revealed the pattern of Fig. 1(a). This experiment was now repeated five times (Table 1), which shows the relative distribution of [^{14}C]arginine in several different 'precursor' preparations before and after trypsin treatment.

Fig. 2. Chromatography on DEAE-cellulose of a limited tryptic hydrolysate of [^{14}C]arginine-labelled 'pro-albumin'

All details are as described for Fig. 1(b), except that the 'proalbumin' preparation consisted of 350 μg of protein (sp. radioactivity 245 000 d.p.m./mg) labelled with L-[U- ^{14}C]arginine as described in the Materials and Methods section. The identification of fractions I, II, III and IV is described in the text. $\circ$, E_{280} ; $\bullet$, ^{14}C radioactivity.

These experiments were repeated with [^{14}C]lysine as label, but no sign was seen of a labelled small peptide in four separate attempts. [^{14}C]Leucine and [^3H]methionine also failed to appear in the peptide.

Electrofocusing experiments

The products of tryptic digestion of precursor were examined by electrofocusing in pH gradients. After the trypsin inhibitor was added, 25–50 mg of serum albumin was added to the reaction mixture and passed through Sephadex G-200, with 10 mM-Tris-HCl, pH 7.9, as eluent. Three or four peak tubes containing approx. 15–25 mg of protein were pooled and placed on a pH gradient of pH 5–7 in 6 M-urea. The results are shown in Figs. 3(a) (untreated precursor) and 3(b) (after tryptic digestion). Fig. 3(b) shows that the ^{14}C -radioactivity of the labelled precursor and the E_{280} of the marker albumin have identical isoelectric points, but the distribution of the radioactivity in the two major peaks of protein is different. This appearance is common in that we have

seen it in 17 of 20 experiments. In the other three, the radioactivity and the E_{280} traces followed each other faithfully. We can offer a partial explanation of this phenomenon. We noticed that in the three experiments in which marker albumin and radioactivity (from 'precursor') coincided closely, there was relatively small loss of 'precursor'. We therefore did experiments to see if a more limited trypsin hydrolysis would give us a more exact fit of marker and radioactive content. The results (four experiments) certainly support the idea. Tryptic hydrolysis with 1 μg of trypsin/ml for 30 min or with 0.5 μg of trypsin/ml for 60 min decreased the preponderance of the more acidic radioactive species, which was seen when 'precursor' was incubated for 60 min with 1 μg of trypsin/ml. These experiments suggest that trypsin may be attacking at least two sites in the 'precursor': one very sensitive (which gives rise to albumin species corresponding to the marker) and another, less sensitive, which predominantly gives rise to albumin(s) with a low isoelectric point. Alternatively, the trypsin preparation contains enzyme impurities which attack 'proalbumin'.

Fatty acid binding. It is possible that changes in binding of fatty acids by albumin (and albumin-like proteins) could give rise to these changes in isoelectric points (see Geller *et al.*, 1972). We therefore mixed a sample of 'precursor' with marker albumin and treated the mixture with charcoal (Chen, 1967). This was then electrofocusing. No variation in the relative positions of 'precursor' and marker albumin was seen; the pattern was identical with that shown in Fig. 3(a).

Effect of trypsin on albumin

The experiments described above depend on the assumption that trypsin (at low concentrations) attacks 'precursor' preferentially, leaving the marker albumin relatively unaltered. That this assumption is valid was established in several different ways.

(1) Rat serum albumin (5 mg, labelled with [^{14}C]leucine) was incubated with trypsin in the standard system described in the Materials and Methods section. The reaction was stopped with trypsin inhibitor and 45 mg of ^3H -labelled rat serum added. The mixture was passed through Sephadex G-200 and the albumin fraction electrofocusing in a pH gradient of pH 5–7. The ^3H , ^{14}C and E_{280} traces coincided perfectly.

(2) The same procedure was used and the proteins were chromatographed on DEAE-cellulose. There was no separation of the two labels and the E_{280} .

(3) Serum albumin was labelled with [^{14}C]arginine. It was chromatographed on DEAE-cellulose and subjected to trypsin as described above. No release of small peptides or free amino acids was detected.

Table 1. *Distribution of radioactivity before and after trypsin hydrolysis of 'precursor' labelled with [^{14}C]arginine*

'Precursor' (derived as described in the Materials and Methods section) contained 18.4 d.p.m./ μg of protein. It was treated with trypsin in the presence of 5 mg of carrier albumin, and then the peptide was separated by chromatography on Sephadex G-25 and the albumin by DEAE-cellulose chromatography as described in the Materials and Methods section.

Expt. no.		^{14}C radioactivity (d.p.m.)		
		In starting material	In products	Difference
1	'Precursor'	9750	Nil	-9750
	Albumin	1080	8550	+7470
	Peptide	Nil	915	+915
2	'Precursor'	153 000	54 000	-99 000
	Albumin	18 000	114 000	+96 000
	Peptide	Nil	3750	+3750
3	'Precursor'	40 000	21 000	-19 000
	Albumin	4500	26 250	+21 750
	Peptide	Nil	2400	+2400
4	'Precursor'	10 500	4350	-6150
	Albumin	1200	6450	+5250
	Peptide	Nil	300	+300
5	'Precursor'	11 490	945	-10 545
	Albumin	1350	10 500	+9150
	Peptide	Nil	750	+750

Fig. 3. *Isoelectric fractionation of 'proalbumin' before (a) and after (b) limited trypsin treatment*

The details of the samples, of trypsin treatment and additions of trypsin inhibitor were as in Fig. 1 (except that 'precursor' was 54 μg of protein; sp. radioactivity 350 000 d.p.m./mg). At the end of incubation, the mixture was subjected to electrofocusing in 6M-urea, pH range 5-7 (see the Materials and Methods section). $\circ$, E_{280} ; $\bullet$, ^{14}C radioactivity; $\blacksquare$, pH.

EXPLANATION OF PLATE I

Radioautograms of peptide 'maps' derived from (a) serum albumin and (b) 'proalbumin'

The two radioautograms were prepared as described in the Materials and Methods section. The proteins were labelled with L-[U- 14 C]arginine and L-[U- 14 C]lysine. Continuous lines enclose ninhydrin-stained spots, which for the 'proalbumin' peptide 'map' (a) are derived predominantly from the marker (unlabelled) albumin, which was added to the 'proalbumin' preparation before the proteolytic step. The darkened area (arrow) seen in the 'proalbumin' peptide 'map' does not appear in (a), and may be the peptide referred to in Table 1.

(4) The amount of 'precursor' was kept constant at 70 μ g of protein and the addition of carrier albumin was varied between 5 and 25 mg during trypsin treatment. No effect on the transformation of 'precursor' was seen.

We conclude that trypsin at low concentrations does not perceptibly affect the serum albumin marker, but that it does indeed attack some sensitive site in the 'precursor' protein.

Peptide 'mapping' of 'precursor'

Leucine-labelled 'precursor'. In the radioautograph of a hydrolysate of 'precursor' mixed with marker serum albumin, only the neutral peptides are radioactive and those which are seen as radioactive correspond to ninhydrin-stained spots on the paper. This radioautograph was indistinguishable from that obtained from pure serum albumin or from that obtained from a mixture of carrier albumin and 'precursor' subjected to limited tryptic hydrolysis and then passed through Sephadex G-200 before peptide 'mapping'.

In another experiment [^{14}C]leucine-labelled 'precursor' was mixed with ^3H -labelled marker albumin and a peptide 'map' prepared. The spots were cut out and their ^{14}C and ^3H radioactivity contents determined. The $^{14}\text{C}/^3\text{H}$ ratio was constant in every one of the 25 leucine-labelled peptides. This experiment was controlled by mixing ^{14}C -labelled albumin and ^3H -labelled albumin, preparing a peptide 'map' and comparing the $^{14}\text{C}/^3\text{H}$ ratios of the radioactive peptides. They were constant.

Arginine- and lysine-labelled 'precursor'. Since the basic peptides were scarcely labelled by [^{14}C]leucine, we next prepared 'precursor' and serum albumin from rats dosed with a mixture of these two ^{14}C -labelled amino acids. 'Precursor' was mixed with unlabelled carrier albumin and the radioautograph of the peptide 'map' (Plate 1b) is indistinguishable from that of the correspondingly labelled albumin (Plate 1a), except for one radioactive spot in the 'precursor' preparation, which fails to correspond with any ninhydrin-stained spot on either chromatogram. One might infer that this single peptide is identical with that detected by column chromatography (Table 1), but the real point is the striking similarity of Plates 1(a) and 1(b).

We conclude that 'precursor' and serum albumin are to all intents identical by these tests.

Antibody precipitation of 'precursor'

The implication of previous studies of antibody reactions with intracellular albumin fractions was that the bulk of the radioactivity precipitated might

arise from simple contamination of the antibody precipitate (Schreiber *et al.*, 1969; Judah & Nicholls, 1971a). We decided to see if anti-albumin was able to distinguish between albumin and 'precursor', and whether 'precursor' was simply adsorbed to the antibody precipitate.

Table 2 illustrates an experiment using the 'double-antibody' technique described in the Materials and Methods section. This shows that anti-albumin cannot distinguish between 'precursor' and albumin.

A second experiment using the antibody-titration method (described in the Materials and Methods section) supported this conclusion. The antibody precipitates contained between 15 and 170 μ g of carrier albumin (depending on the amount of antibody added), but the apparent specific radioactivity of the precipitated protein remained constant. Since 90% of the radioactivity arose from precursor, and more than 90% of the protein was unlabelled albumin, it may be concluded that anti-albumin does not distinguish between 'precursor' and albumin.

'Precursor' pool in liver

From the double-antibody experiment (Table 2) it can be calculated that, since the anti-albumin was saturated by 9–10 μ g of albumin, the 'specific radioactivity' of the bound albumin was about 54 d.p.m./ μ g (tube 2). Therefore the total protein present was $900/54 = 16.5 \mu$ g, of which 10 μ g was added albumin. The balance of 6.5 μ g would thus be precursor, with a specific radioactivity of 140 000 d.p.m./mg. Since the crude antibody precipitate contained 180 000 d.p.m. in 'precursor' we can conclude that 1.30 mg of 'precursor' was present in the microsomal fraction derived from 35 g of rat liver, or 37 μ g/g of liver.

Table 2. Reaction of 'precursor' with anti-albumin-anti-globulin complex

Purified 'precursor' (labelled with [^{14}C]leucine, 1000 d.p.m.) was added to anti-(rat albumin)-anti-(rabbit globulin) complex, which had previously been allowed to react with unlabelled albumin as shown in column 2 of the table. The amount of anti-albumin present was enough to bind approx. 9 μ g of serum albumin. The preparation of the antibody complex and purification of precursor are described in the Materials and Methods section.

Tube no.	Albumin added (μ g)	^{14}C radioactivity in antibody ppt. (d.p.m.)
1	0	900
2	10	510
3	100	38

A repetition of the experiment with an entirely separate microsome preparation gave a value of 1.4 mg of 'precursor' in the microsomal fraction derived from 35 g of rat liver, an average of 40 μ g/g of liver, but a third average result was only 20 μ g/g of liver. This means that 30–40% of the antibody-precipitable 'albumin' fraction of rat liver microsome preparations is apparently 'precursor'. In contrast, the antibody-precipitable 'albumin' fraction of whole liver is 90% albumin (Judah & Nicholls, 1971a), so one assumes that 'precursor' is concentrated in the microsomal fraction. In any case, the liver content of 'precursor' has to be corrected upwards. From cytochrome P_{450} measurements, it seems that about 60% of the microsomal fraction (from the initial homogenate) is lost on processing. Further, we find that if we add labelled 'precursor' to microsomal preparations after deoxycholate treatment (Judah & Nicholls, 1971a), some 50% of the 'precursor' disappears during the time taken to clarify the extract before antibody precipitation. Therefore the values for microsomal 'precursor' content quoted above may well be 2.5–5 times too low, i.e. the real value may be 100–200 μ g of 'precursor' in the total microsome preparation from 1 g of rat liver.

The turnover time for the 'precursor' could therefore lie between 6 and 12 min, assuming a secretion rate of 1 mg of albumin/h per g of liver. It is prudent to remember that the 'albumin' content of rat liver (Gordon & Humphrey, 1961) and most probably that of 'precursor' might vary widely.

Conclusions

We believe that enough evidence is presented here to establish the existence of a protein precursor to serum albumin in rat liver. We suggest that this material be known as 'proalbumin' by analogy to pro-insulin (Steiner *et al.*, 1967; Grant & Coombs, 1970).

It is not by any means certain, however, that proteolytic cleavage of 'proalbumin' is the physiological mechanism by which serum albumin is produced by the liver cell. One alternative, for example, could be the masking of positively charged groups by the addition of acyl groups (to, e.g., lysine in the protein). We have not been able to acylate 'proalbumin' by interaction with acetyl-CoA or palmitoyl-CoA *in vitro* in the presence of liver microsomal preparations (our unpublished work), but perhaps other acyl groups could be involved (e.g. sugar acids). This uncertainty is the penalty paid for our ignorance of the detailed structure of rat serum albumin.

Protein precursors to enzymes are well known and need not be remarked on here, though an interesting example has been reported by Vaes (1972), who has described the apparent conversion of a procollagenase into collagenase by trypsin. In these experiments the trypsin activated a fibroblast enzyme, which in turn acted on procollagenase. Milstein *et al.* (1972)

have, however, identified what seems to be a precursor for immunoglobulins, which is converted into its product by limited tryptic hydrolysis. They suggest that the small peptide thus severed from the molecule may act as a 'recognition signal' to indicate a protein intended for export. We favour the idea put forward in a previous paper (Geller *et al.*, 1972) that the existence of a 'proalbumin' might assist albumin secretion in a coupled conversion-secretion. That the existence of a proenzyme (or other precursor protein) is limited to proteins for export, is also rendered doubtful by the finding of Lazarow & de Duve (1971) that the peroxisomal catalase is synthesized in a form recognizable by antibody but devoid of enzyme activity for a considerable time. It will be of interest to learn by what mechanism the inert proenzyme attains its activity.

We should face the possibility that the existence of protein precursors is a general phenomenon in eukaryotic cells, enabling them to control protein production without calling nuclear mechanisms into action. If this were so, the interpretation of some well-established results might be questioned. For instance, if an agent is shown, apparently, to induce the synthesis of a protein *de novo* (see Schimke & Doyle, 1970, for some specific instances), it is possible that the effect of that agent is to prevent the destruction of precursor. This effect on a precursor could not be distinguished from effects on apparent synthesis of the product *de novo*, and hence the foundations of the interpretation would disappear.

Whatever the truth of the matter, the possible occurrence of protein precursors should be borne in mind especially by those working with a combination of immunological and radiochemical methods.

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