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Intracellular Distribution of Serum Albumin and its Possible Precursors in Rat Liver

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1. The fractionation of intracellular albumin labelled with radioactive L-leucine was studied in rat liver by means of isoelectric focusing. 2. Isoelectric fractionation was compared with ion-exchange chromatography for purification of radioactive intracellular albumin obtained by antibody precipitation. Similar results were obtained with both methods of separation. Purified albumin contains only a minor amount of the radioactivity. The remainder is associated with albumin-like protein(s). 3. The albumin-like protein has the properties of a precursor of plasma albumin. 4. The distribution and turnover of radioactive albumin in rough and smooth microsomal fractions and in a Golgi-rich fraction were studied. 5. It is concluded that newly synthesized albumin, as such, appears only momentarily if at all in any intracellular structure before its appearance in the plasma. 6. It is also concluded that the rate-limiting step in the secretion of plasma albumin is the conversion of precursor(s) into albumin. We can find no evidence to suggest that there is any significant transport of albumin, as such, during the course of secretion.

We have observed, in agreement with Schreiber *et al.* (1969), that intracellular serum albumin purified by antibody precipitation after an amino acid-incorporation experiment may contain large amounts of radiochemical impurity (Judah & Nicholls, 1971*a*). Our work, with albumin purified by ion-exchange chromatography, suggests that a polypeptide precursor (but not intracellular serum albumin) may be the source of extracellular serum albumin (Judah & Nicholls, 1971*b*).

In the present paper we have studied the relationship of the radiochemical impurity (which may be derived from the hypothetical precursor) to intracellular and extracellular serum albumin. We report results of experiments concerned with alternative methods of purification, the kinetics of labelling and radiochemical balance of albumin fractions and the intracellular radiochemical impurity. This work lends strong support to the hypothesis that the radiochemical impurity may indeed represent an intracellular pool of polypeptide precursor(s) to extracellular serum albumin.

Materials and Methods

Materials

Animals, radioactive materials and counting, albumin and antialbumin preparations were as previously described (Judah & Nicholls, 1970, 1971*a*). Chemicals were AnalaR grade; sucrose for isoelectric focusing was purchased from Mann Research Laboratories Inc., New York, N.Y., U.S.A. Ampholine carrier ampholyte was obtained from LKB, Stockholm, Sweden. Rat serum albumin from liver extracts was purified by antiserum precipitation and ion-exchange chromatography on CM-cellulose as previously described (Judah & Nicholls, 1971*a*). Labelled albumin was prepared from rat serum as described by Debro *et al.* (1957) and precipitated by addition of 2vol. of diethyl ether.

Injection of radioisotopes

Female rats (150–180g body wt.) under light ether anaesthesia were injected intravenously by the tail vein with radioisotope in 150mM-NaCl. Unless otherwise described the animals received either 20μCi of L-[1-^{14}C]leucine or 100μCi of L-[4,5-^{3}H]leucine. They were killed 12.5min after the injection. For double-label experiments the [^{3}H]leucine was given first, followed 50min later by [^{14}C]leucine; the animal was killed 12.5min after the ^{14}C injection. For other experiments, the rats were given 6.7μCi of L-[1-^{14}C]-leucine/100g body wt. by intravenous injection.

Preparation of deoxycholate extracts for Sephadex chromatography and isoelectric focusing

Immediately after death, the liver was removed and run through an ice-cold tissue press (Harvard Apparatus Co., Dover, Mass., U.S.A.). The pulp

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expressed from one liver was homogenized in a loose-fitting glass homogenizer (Blaessig Glass Co., Rochester, N.Y., U.S.A.) in 50ml of ice-cold 30mM-tris-HCl (pH8)-0.25M-sucrose. The homogenate was centrifuged for 10min at $6000g_{av.}$. The supernatant was centrifuged at $164000g_{av.}$ for 60min and the combined microsomal pellets were suspended in 5mM-tris-HCl, pH8.0, containing 2.5mg of carrier rat serum albumin. The suspension was adjusted to a final deoxycholate concn. of 0.5% (w/v), which was enough for maximal yield of soluble radioactive products in these experiments. The mixture was then centrifuged at $164000g_{av.}$ for 60min in the 50 Ti or 65 rotor of the Beckman L2-65B centrifuge.

Isoelectric fractionation (electrofocusing)

An LKB column (110ml vol.) was used. The anode solution was 24g of sucrose dissolved in 28.4ml of 260mM-H_2SO_4. This was run into the column to a level 25mm above the bottom of the central tube. Above this was generated 110ml of a sucrose density gradient (Vesterberg & Svensson, 1966) containing the protein to be analysed. Protein was left out of the bottom and top 13.8ml portions of the gradient to obviate contact with the electrode solutions. The dense solution for the gradient consisted of 28g of sucrose dissolved in 42ml of 2.14% (w/v) of the appropriate LKB ampholyte solution. The light solution was 0.5% (w/v) ampholyte and contained the protein mixture or extract. The light electrode solution was 250mM-NaOH, which was layered above the sucrose gradient. The starting voltage was about 400V, which was raised to about 1000V after some 12h. Power dissipation was not allowed to exceed 2W and the column was kept at 2°C. Within 36h the current reached a minimum and the process was complete. The column was drained into a fraction-collector at about 2ml/min, 1ml fractions being collected.

It was found that excessive precipitation disrupted the column when attempts were made to electrofocus crude deoxycholate extracts. The precipitation could be minimized by first passing the extract through Sephadex G-200, and successful isoelectric fractionation was then possible.

Chromatography on Sephadex G-200

The column dimensions were 25mm × 275mm, void volume 50ml. The water-jacketed column was kept at 2°C. A sample (5.0ml) of deoxycholate extract was applied and eluted with 10mM-tris-HCl buffer, pH8.0, at a flow rate of 0.5ml/min; 2.0ml fractions were collected. They were pooled as needed for isoelectric fractionation.

Assay of column fractions

The E_{280} was measured (10mm light-path) with water as reference. Radioactivity was measured as described by Judah & Nicholls (1970, 1971*a*). pH was measured at room temperature (20°C). It should be remembered that these pH values will be about 0.2 unit lower than those at the temperature of the columns (2°C).

Double-labelling experiments

Since we were interested in newly synthesized pools of intracellular albumin, we devised a simple method for their detection. It is known that secretion of newly synthesized albumin in the rat is complete between 40 and 60min after injection of labelled L-leucine (Peters, 1962). Maximal labelling of intracellular albumin is observed within 10–15min (Judah & Nicholls, 1971*b*). About 50min after a first label, therefore, the labelled intracellular albumin will be that which is not exported (or which is slowly synthesized). If a second label is now administered and the animal is killed at 12.5min, the second label will be present in relatively larger amounts in that albumin which is about to leave the liver. This albumin will, by the same token, be poor in the first label. Therefore a high $^{14}C/^{3}H$ ratio is indicative of rapid turnover.

One must distinguish between newly synthesized (but not secreted) albumin and that which is newly secreted, since the latter will be heavily labelled by the first label, whereas the second label (after 12.5min) will scarcely have had time to emerge from the liver in serum albumin. In practice therefore a first label of 100μCi of L-[4,5-^{3}H]leucine is given intravenously. Then 50min later, 20μCi of L-[1-^{14}C]leucine is given intravenously. At 12.5min after the injection of [^{14}C]leucine, the animal is killed and further operations are carried out as described in the various experiments. We have used similar methods in our slice experiments (Judah & Nicholls, 1971*b*).

One further point to remember about these experiments is the relatively rapid disappearance of the labelled amino acid from the plasma after intravenous injection. Peters (1962) reports that almost all the free leucine radioactivity has disappeared from the plasma within 10min. We have confirmed these findings.

This method is specially useful if one is looking for a small pool of material that turns over rapidly, but which might be concealed because of the presence of a relatively large amount of protein that turns over slowly, e.g. small compartments in the endoplasmic reticulum or small numbers of active cells in a heterogeneous tissue.

Microsomal subfractionation

This was done by a modification of the method described by Tata (1969). All sucrose solutions

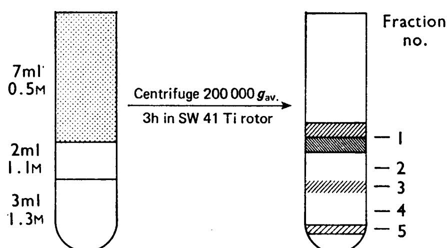


Fig. 1. *Discontinuous-density-gradient separation of submicrosomal fractions*

The position of the five fractions obtained is shown in the diagram as they appear in the tubes of the SW 41 Ti rotor after 180min centrifugation at $200000g_{av.}$ under the conditions described in the Materials and Methods section. Fraction 1 contains a preponderance of smooth endoplasmic reticulum (low RNA/protein ratio in Table 3). Fraction 5 contains a preponderance of rough endoplasmic reticulum (high RNA/protein ratio in Table 3); the intermediate fractions are presumably intermediate in composition.

contained 50mM-tris–HCl, pH7.6, 50mM-KCl and 10mM-$MgCl_2$. All operations were done at 0–2°C. Homogenates were prepared in 3vol. of 500mM-sucrose, centrifuged at $7000g_{av.}$ for 10min, and the supernatants were carefully removed. Of each supernatant 7.0ml, containing about 20mg of microsomal protein, was layered over a discontinuous density gradient consisting of 3.0ml of 1.3M-sucrose and 2.0ml of 1.1M-sucrose in three to six tubes and centrifuged in the SW 41 Ti rotor for 3h at $200000g_{av.}$ on a Beckman L2-65B centrifuge. Fig. 1 shows the results of this separation, and Table 3 gives RNA/protein ratios for the five fractions obtained. Fraction 1 (with a low RNA/protein ratio) contains mostly the smooth microsomal fraction; fraction 5 (with a high ratio) is mainly rough microsomal fraction.

For experiments concerned with the distribution of radioactive albumin between smooth and rough microsomal fractions, fractions 1–3 inclusive were designated smooth. These were removed from the centrifuge tubes with a syringe and bent stainless-steel needle and combined. Rough fractions 4 and 5 then were aspirated off after fraction 5 had been separated from the underlying glassy pellet by swirling the tube gently. Individual fractions when needed were removed with a syringe in the same manner, but collected and kept separately. Antibody precipitation and ion-exchange chromatography of the precipitated albumin were done on deoxycholate extracts of microsomal fractions and subfractions as previously described (Judah & Nicholls, 1971*a*,*b*). Total microsomal fractions were obtained from the postmitochondrial supernatant by centrifugation at $164000g_{av.}$ for 60min. Separation of a Golgi-rich fraction was done by the method of Leelawathi *et al.* (1970). This was modified as follows. (1) The homogenate was centrifuged at $6000g_{av.}$ for 10min to yield a 'post-mitochondrial supernatant' instead of using a 'post-nuclear supernatant' as described by these authors. (2) The first density-gradient centrifugation was done in the SW 41 Ti rotor at $100000g_{av.}$ for 60min, instead of in a fixed-angle rotor. (3) The second density-gradient centrifugation was also done in the SW 41 Ti rotor at $200000g_{av.}$ for 60min.

RNA was determined by the orcinol method (Schneider, 1957); 1mg of pentose was assumed to represent 3.76mg of RNA.

Protein was determined by the biuret method (Layne, 1957) or by that of Lowry *et al.* (1951). Rat serum albumin was used as reference standard.

Results and Discussion

Isoelectric fractionation of serum albumin

Isoelectric points. The isoelectric point reported for rat serum albumin is 4.6 (Anderson *et al.*, 1959). However, with rat serum albumin isolated by the method of Debro *et al.* (1957), purified as described by Judah & Nicholls (1971*a*), we find an isoelectric point of 5.7 by the method of isoelectric fractionation (Vesterberg & Svensson, 1966). We have also electrofocused ^{3}H-labelled whole rat serum in the presence of ^{14}C-labelled marker serum albumin, separated by the method of Debro *et al.* (1957). The ^{3}H- and ^{14}C-labelled albumin samples coincided at a pH band of 5.7. Finally, we electrofocused another sample of fresh ^{14}C-labelled whole rat serum, without any addition of carrier or marker albumin in case such an addition might influence the results. The position of albumin was identified by its E_{280} and by antibody precipitation. The apparent isoelectric point was 5.7.

Perhaps the explanation for these results is that suggested by Valmet (1970) for human serum albumin, namely that the isoelectric point of albumin may in part be determined by the binding of fatty acids, and thus may vary considerably. We mixed 10mg of rat serum albumin with 5.0mg of stearate (adjusted to pH8.5 with tris base), left the mixture for 30min at 38°C, and then adjusted the pH to about 5. The mixture was centrifuged ($20000g_{av.}$) to clear and was then electrofocused. The albumin separated into two equal fractions, one with isoelectric point at pH4.7 and the other at pH5.7. Stearate was chosen arbitrarily and has no particular significance. A control (no stearate) ran to pH5.7. This supports Valmet's (1970) suggestion. One must also bear in mind the possibility that the method of

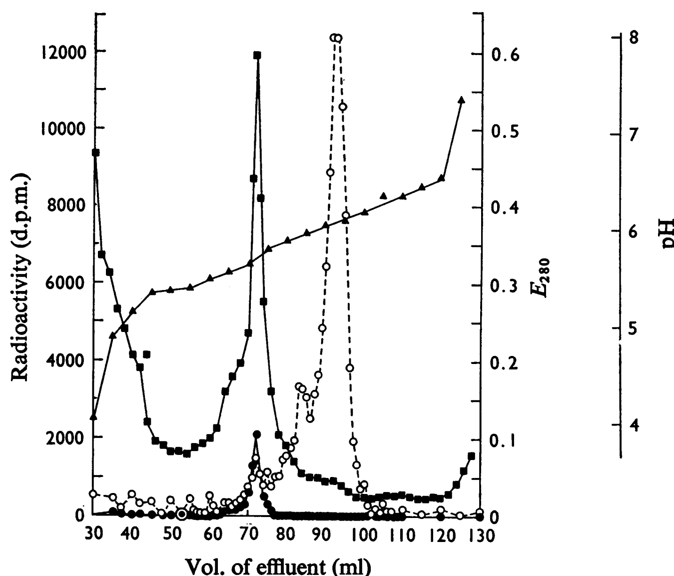


Fig. 2. *Separation of radioactive impurity from intracellular albumin by isoelectric focusing*

A ^{3}H-labelled albumin fraction (125000d.p.m.) obtained by antiserum precipitation from a deoxycholate extract (see the Materials and Methods section) was mixed with 15000d.p.m. of ^{14}C-labelled serum albumin (1500d.p.m./mg). The mixture was electrofocused in pH5–7 Ampholine carrier ampholyte at 1000V for 41h. ●, ^{14}C; o, ^{3}H; ■, E_{280}; ▲, pH.

electrofocusing itself strips bound anions off the albumin during the long period of fractionation, and thus gives an isoelectric point of 5.7 instead of the 4.6 reported by Anderson *et al.* (1959).

Separation of intracellular albumin. Intracellular albumin was labelled with [^{3}H]leucine and separated from rat liver by antibody precipitation. The antibody precipitate was dissociated as described by Judah & Nicholls (1971*a*) and the soluble products were electrofocused in the presence of ^{14}C-labelled carrier serum albumin. Fig. 2 shows that the ^{3}H-labelled proteins separate into two main components: a major portion of the radioactivity appears at pH6.0–6.1, and the albumin marker appears at pH5.7 associated with a very minor amount of ^{3}H label derived from the antibody precipitate. This confirms by a new means the findings of Schreiber *et al.* (1969) and of Judah & Nicholls (1971*a*). The absence of serum albumin from the highly labelled material at pH6.0–6.1 was confirmed. Samples from tubes 90–95 (see Fig. 2) were pooled, and unlabelled albumin was added and precipitated with anti-albumin. The precipitate, containing almost all the radioactivity of these fractions, was subjected to ion-exchange chromatography on CM-cellulose (Judah & Nicholls, 1971*a*). The carrier albumin was isolated and found to contain no radioactive label.

The protein(s) species appearing at about pH6.0 (Fig. 2) was isolated in separate experiments, the contents of the peak tubes being pooled and mixed with non-radioactive albumin. Chromatography on Sephadex G-200 failed to separate the radioactive material from the marker albumin. This indicates little difference in size between the two.

Isoelectric fractionation of deoxycholate extracts; use of the double-labelling method. We were encouraged by our success in purification of labelled intracellular albumin from antibody precipitates to attempt direct separation of this protein from deoxycholate extracts of microsomal fractions by isoelectric fractionation without prior use of antibody. At the same time, we used the technique of double labelling (see the Materials and Methods section) to examine the behaviour of microsomal albumin and related proteins.

As explained above (see the Materials and Methods section), deoxycholate extracts of microsomal fractions precipitate in the electrofocusing column unless previously passed through Sephadex G-200. A preliminary experiment was done with a deoxycholate extract. This contained some 90% of the total radioactivity of the microsomal fraction with a ^{14}C/^{3}H ratio of 0.096. The extract was chromatographed on Sephadex G-200 with the result shown in Fig. 3,

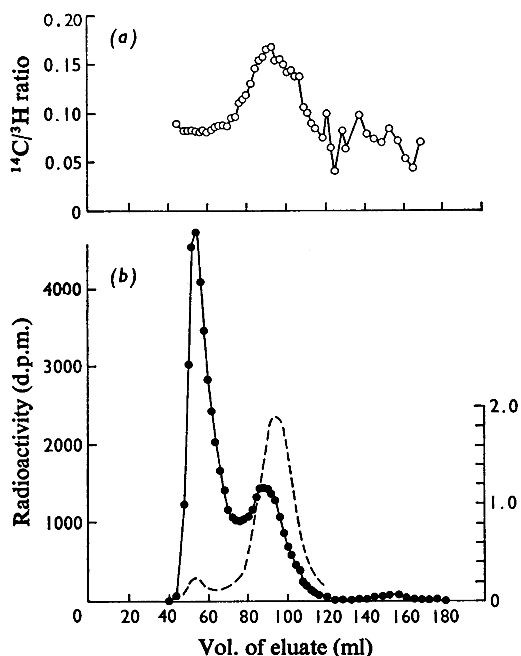


Fig. 3. *Chromatography of deoxycholate extract of double-labelled microsomal fraction on Sephadex G-200*

A deoxycholate extract containing 93300d.p.m. of ^{14}C and 973000d.p.m. of ^{3}H (i.e. $^{14}C/^{3}H$ ratio = 0.096) was applied to the Sephadex G-200 column (see the Materials and Methods section). The elution pattern is compared with the profile given by a rat serum albumin standard (measured by E_{280}) in a separate experiment. ●, ^{14}C; o, $^{14}C/^{3}H$ ratio; ----, E_{280}.

which also includes the E_{280} trace of marker albumin. About 15% of the radioactivity applied to the column emerged in the position expected of molecules of the size of albumin. The $^{14}C/^{3}H$ ratio also rose to 0.15 (as shown in Fig. 3*a*). The tubes with the high $^{14}C/^{3}H$ ratio contained about 95% of the radioactivity precipitated by antialbumin from the original extract. The crude antibody precipitate gave a $^{14}C/^{3}H$ ratio of 1.03, and the albumin separated from it by ion-exchange chromatography had a $^{14}C/^{3}H$ ratio of 0.5.

These results were reproducible. The $^{14}C/^{3}H$ ratio of the original extracts varied from 0.096 to about 0.3, but it always doubled (approximately) in the region where intracellular albumin emerged. Isoelectric fractionation of the eluate from Sephadex G-200 columns was next attempted to see if purification of intracellular albumin could be effected without the use of antibody precipitation and to determine in particular whether we could separate the protein(s) with high $^{14}C/^{3}H$ ratio, which always accompanied

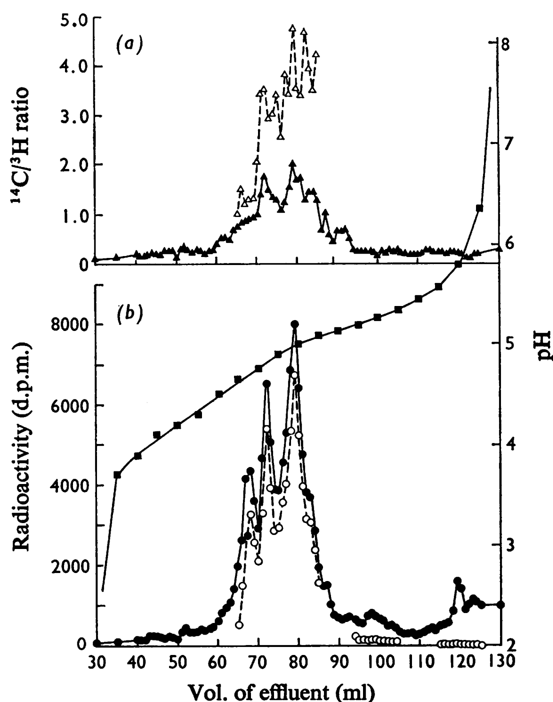


Fig. 4. *Isoelectric focusing of the 'albumin fraction' of double-labelled microsomal fraction*

A deoxycholate extract of microsomal fraction, containing 411000d.p.m. of ^{14}C ($^{14}C/^{3}H$ ratio = 0.31) was chromatographed on Sephadex G-200 (as described in Fig. 3). The combined Sephadex eluate obtained from 72–112ml (see Fig. 3) was mixed with pH4–6 Ampholine carrier ampholyte (see the Materials and Methods section). A small precipitate was removed by centrifugation and the supernatant fluid, containing 150000d.p.m. of ^{14}C ($^{14}C/^{3}H$ ratio = 0.59), was electrofocused at 900V for 59½h. Samples of appropriate fractions were treated with excess of antiserum in the presence of carrier albumin. ●, ^{14}C radioactivity (d.p.m.); o, radioactivity of antiserum precipitate; ▲, $^{14}C/^{3}H$ ratio before precipitation of antiserum; △, $^{14}C/^{3}H$ ratio of antiserum precipitate; ■, pH.

intracellular albumin in antibody precipitates. A deoxycholate extract of labelled microsomal fraction was prepared and passed through Sephadex G-200. Its $^{14}C/^{3}H$ ratio, originally 0.31, rose to 0.59 in the relevant fractions of the Sephadex eluate. Fig. 4 shows the results of isoelectric fractionation. Three peaks are observed, at pH4.7 (peak I), pH4.8 (peak 2) and pH5.0 (peak 3). Peak 1 (pH4.7) was identified as serum albumin by separate experiments with

Table 1. *Comparison of isoelectric fractionation and ion-exchange chromatography in the separation of intracellular albumin and related proteins*

A single deoxycholate extract of microsomal fraction was used as starting material. The isoelectric-fractionation results are derived from Fig. 4. Values in parentheses are the $^{14}C/^{3}H$ ratios in antibody precipitates of the fractions removed from the LKB column. Antibody precipitation and ion-exchange chromatography were done as described by Judah & Nicholls (1971*a*). Other details are given in Fig. 4.

Method of purification	Fraction	Ratio $^{14}C/^{3}H$
—	Crude extract	0.31
Sephadex chromatography	Eluate	0.59
Isoelectric fractionation of the above eluate	Albumin (peak 1)	0.87–0.91 (1.2–1.3)
	Peak 2	1.70 (3.5)
	Peak 3	2.0 (4.7)
Antibody precipitation and ion-exchange chromatography	Albumin	1.2
	Albumin-like proteins	3.2

marker albumin. Most of the radioactivity of the peaks at pH4.8 and 5.0 is precipitated with anti-albumin in the presence of carrier albumin (as shown in Fig. 4), and these peaks are taken to represent the albumin-like proteins found in antibody precipitates. Fig. 4(*a*) shows a substantial rise in the $^{14}C/^{3}H$ ratio, corresponding to peaks 2 and 3, and the antibody precipitates of these fractions (in Fig. 4*a*) show an even larger increase.

In Table 1 we compare the results of isoelectric fractionation with those obtained by antibody precipitation followed by ion-exchange chromatography of the same microsomal extract. The results are strikingly similar. These results are reasonably reproducible: some variation is seen, e.g. in relative heights of the peaks (one may become a shoulder to another) and in position (shifts of 0.05–0.10 pH unit). But the pattern was the same in three separate runs.

The isoelectric point found for serum albumin in this experiment (at pH4.7) differs markedly from that found in Fig. 2. But it is the same as that found by us for albumin after incubation with stearate and we think it possible that anions (perhaps fatty acids) in the deoxycholate extract have become firmly bound to the albumin and related proteins.

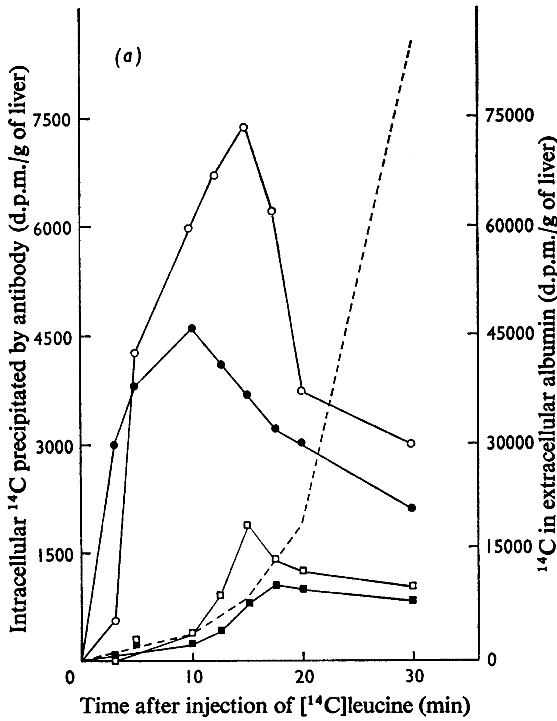
In any event, the method of isoelectric fractionation confirms the results of Schreiber *et al.* (1969) and of our own earlier work (Judah & Nicholls, 1971*a*) about the occurrence of highly radioactive protein contaminants in antibody precipitates of intracellular albumin. Our experiments also show that these albumin-like proteins are turning over rapidly, as judged by the double-label experiments, and they will be referred to later.

To summarize, isoelectric fractionation was used for two purposes: first, to provide an alternative method to ion-exchange chromatography for the purification of albumin in the antibody precipitate, and secondly, to fractionate the soluble proteins in crude deoxycholate extracts of microsomal fractions to look for possible precursors of albumin. To this end, we also applied our method of double labelling for the identification of proteins that are rapidly exported from the liver, or which are turning over quickly.

The results indicate that we succeeded. The proteins of the antibody precipitate were separated into albumin, which contained only a small amount of radioactivity, and other protein(s), which contained most of the radioactivity of the antibody precipitate. Isoelectric fractionation of the crude deoxycholate extract without prior antibody separation showed the presence of the proteins that appeared in the antibody precipitate. Intracellular albumin appeared to turn over relatively slowly, but the other(s) (containing most of the radioactivity) turned over much more rapidly. No other soluble proteins in the microsomal extract revealed this behaviour.

Intracellular distribution of serum albumin

To look for some intracellular site in which we could detect albumin that might be a precursor of the extracellular protein, we separated smooth and rough microsomal fractions from rats labelled *in vivo* with L-[1-^{14}C]leucine. Fig. 5(*a*) shows that the crude antibody precipitate becomes radioactive very quickly, but that the purified albumin is labelled after a lag period of 10min. The results are given as d.p.m./g of liver so that the relationships can be seen easily and because in any case it is not possible to give a specific radioactivity for the unknown proteins in the antibody precipitate. Fig. 5(*b*) shows the specific radioactivity of the albumin in the smooth and rough microsomal fractions compared with that calculated for newly secreted plasma albumin. The radioactivity of plasma albumin begins to rise at



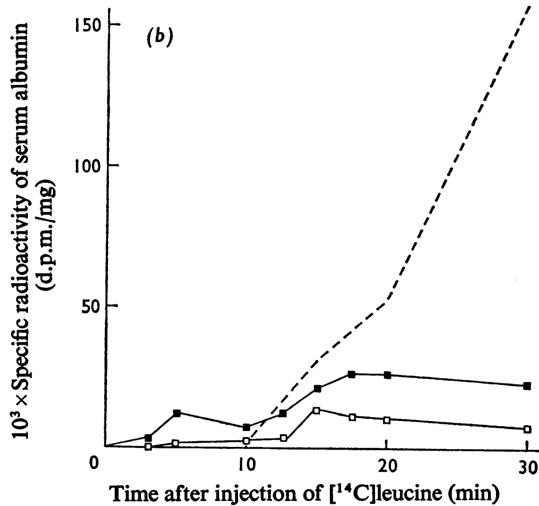


Fig. 5. *Radioactivity of intracellular albumin*

(*a*) Distribution of radioactivity in total antibody precipitate and albumin in smooth and rough endoplasmic reticulum. (*b*) Specific radioactivity of serum albumin in smooth and rough endoplasmic reticulum. Female rats (170g body wt.) were given L-[1-^{14}C]leucine (11.3μCi) by intravenous injection. The results are corrected for recovery of carrier serum albumin added to the microsomal extracts before antibody precipitation. The numbers of observations and extent of variations are recorded in Table 2. Specific radioactivity of serum albumin is given as d.p.m./mg of protein. The plasma albumin radioactivity is calculated on the basis of a pool of 400mg of extracellular albumin, at a rate of secretion of 1mg of albumin/h per g of liver (Judah & Nicholls, 1971*b*). The albumin content (calculated per g of liver) of smooth microsomal fraction was 126±23μg and that of the rough was 38±8μg (means±S.D.). Radioactivity of total antibody precipitate: o, in smooth endoplasmic reticulum; •, in rough endoplasmic reticulum. Radioactivity of albumin: □, in smooth endoplasmic reticulum; ■, in rough endoplasmic reticulum; ----, in plasma.

Table 2. *Comparison of radioactivity of antibody-precipitated protein and purified albumin in smooth and rough microsomal fractions*

The results (means±S.D.) refer to the corresponding times shown in Fig. 5(*a*).

	100×Radioactivity (d.p.m. in smooth fraction/d.p.m. in rough fraction)		
Time (min)	Crude antibody ppt.	Purified albumin	No. of observations
3.0	16± 3.9	Nil	5
5.0	120±38	70± 38	5
10.0	144±20	149± 50	5
12.5	149±31	268± 84	5
15.0	198±31	268±130	5
17.5	195	135	2
20.0	112	122	3
30.0	110	120	1

about the same time as albumin in the smooth microsomal fraction, with a time-lag of 2–3min at most. This suggests that the greater part of the time taken for labelled albumin to appear in the plasma is that required for conversion of labelled precursor into complete albumin. This newly synthesized albumin then must reside in the liver for a maximum of 3min, perhaps in some part of the smooth endoplasmic reticulum.

Table 2 shows the extent of variation of the results reported in Fig. 5(*a*). This method of presentation has been chosen because of the relatively large variation in radioactivity from one rat to another. The relationship of the two fractions to each other was always in the same sense as shown in Fig. 5(*a*) and Table 2 in every one of the more than 30 animals used in these and other experiments.

Table 3. *Distribution of RNA, protein and labelled albumin fractions of endoplasmic reticulum*

The microsomal subfractions were separated in a discontinuous density gradient as described in the Materials and Methods section. The [^{14}C]leucine contents of the albumin fractions are the means of three separate experiments with female rats of about 170g body wt. The ^{14}C/^{3}H ratios are the means of three experiments done as described in the Materials and Methods section. The fraction nos. correspond to those shown in Fig. 1. The antibody precipitate and purified albumin were separated as described in the Materials and Methods section. The lowest RNA/protein ratio indicates a fraction containing a predominance of smooth endoplasmic reticulum; the highest RNA/protein ratio suggests that the fraction consists largely of rough endoplasmic reticulum. The protein and RNA contents are means ± S.D. of results from five experiments.

Fraction no.	Content (mg/g of liver)		Ratio RNA/protein	^{14}C radioactivity (d.p.m./g of liver)		Ratio ^{14}C/^{3}H	
	Protein	RNA		In crude antibody ppt.	In purified albumin	In crude antibody ppt.	In purified albumin
1	3.06±0.4	0.053±0.02	0.017	1790	370	5.28	0.84
2	2.0 ±0.6	0.094±0.04	0.05	1565	248	6.04	0.93
3	1.59±0.4	0.12 ±0.05	0.08	1440	141	6.24	1.32
4	3.27±1.0	0.79 ±0.21	0.24	1775	69	10.51	1.30
5	4.47±1.1	1.38 ±0.43	0.31	1630	244	8.41	1.37
Total microsomal fraction	14.4	2.45	0.17	—	—	7.44	1.08

Table 4. *Radioactivity of antibody-precipitated protein and of albumin of microsomal fraction and of Golgi-rich fraction*

The Golgi-rich fraction of rat liver was separated by the method of Leelawathi *et al.* (1970). The microsomal fractions were obtained from the same livers by centrifugation of the postmitochondrial supernatant at $164000g_{av.}$ for 60min. The [^{14}C]leucine contents of the antibody ppt. and of albumin and the ^{14}C/^{3}H ratios were obtained as described in the Materials and Methods section. The time refers to the time taken between the injection of ^{14}C and the death of the animals.

	Radioactivity (d.p.m./g of liver)							
	Microsomal fraction				Golgi-rich fraction			
	Crude antibody ppt.		Albumin		Crude antibody ppt.		Albumin	
Time (min)	^{14}C	^{14}C/^{3}H	^{14}C	^{14}C/^{3}H	^{14}C	^{14}C/^{3}H	^{14}C	^{14}C/^{3}H
3	3750	—	Nil	—	375	—	Nil	—
12.5	15000	8.0	1720	2.2	3350	7.1	500	2.1

In an attempt to find a subcellular site of the newly synthesized albumin we therefore applied our double-label technique and prepared submicrosomal fractions. The results of these experiments (Tables 3 and 4) show that no single subfraction (including the Golgi-rich fraction) yields radiochemically purified albumin that is markedly enriched in ^{14}C when compared with any other subfraction, or indeed with the total microsomal fraction.

Albumin-like protein in the antibody precipitate

From the results in Figs. 2–5 it is clear that the material that is present in antibody precipitates and is separated from albumin by the various means employed comes nearer in properties to an albumin precursor than anything else yet encountered. As shown in Fig. 5(*a*), its radioactivity rises very rapidly after the injection of labelled leucine and falls at the time when albumin is being released to the plasma. It

Table 5. *Changes in intracellular and extracellular radioactivity accompanying synthesis of serum albumin*

Female rats (170g body wt.) received intravenous injections of 11.3μCi of L-[1-^{14}C]leucine at zero time. At 2min later they received 0.25mg of cycloheximide by intravenous injection and were killed at either 15min or 30min after the [^{14}C]leucine injection. Blood samples were then collected and the livers removed and microsomal fractions separated as described in the Materials and Methods section. The microsomal albumin fractions and plasma albumin were separated as described in the Materials and Methods section. The results (means ± S.D.) are corrected for recovery of carrier albumin added to the microsomal extracts before antibody precipitation. These results in turn are corrected (where indicated) for an assumed recovery of microsomal fraction of 40%. Numbers in parentheses denote the numbers of observations. Δ d.p.m. is the loss (−) or gain (+) in the period.

	Microsomal radioactivity (d.p.m./g of liver)		
Time (min)	Crude antibody ppt.	Purified albumin	Plasma albumin
15	8700±900 (5)	3200±855 (5)	8200±2160 (5)
30	3580±980 (5)	1330±685 (5)	27000±7450 (5)
Δd.p.m.	−5120	−1870	+18800
d.p.m. corr. for microsomal recovery	−12800	−4675	+18800

is the most rapidly turning-over component and constitutes a major fraction (10–20%) of the total radioactivity of extracts of rat liver microsomal fraction at the time of maximal labelling.

Glaumann (1970) found that administration of cycloheximide (1mg/kg body wt.) 2min after [^{14}C]-leucine decreased by only 20% the total incorporation of leucine into plasma albumin (measured at 60min). We have found rather larger inhibitions (40–50%), but in any event 2min exposure of rats to [^{14}C]leucine permits an extensive incorporation of label into plasma albumin. Cycloheximide allows one in effect to administer a pulse of labelled amino acid. With this technique we have compared the changes of radioactivity in the crude antibody precipitate and in the purified albumin from microsomal fraction with the appearance of radioactive albumin in the plasma in the time-interval between 15 and 30min after the administration of [^{14}C]leucine. Table 5 shows that neither the crude antibody precipitate nor the purified albumin completely accounts for the amount of labelled plasma albumin secreted during the 15min studied. However, if these results are corrected for a 40% recovery of microsomal fraction from liver homogenates prepared by our methods, an approach to balance can be struck between the changes in the radioactive protein(s) in the crude antibody precipitate and the radioactive plasma albumin secreted in the period studied.

Conclusions

We propose that the material that co-precipitates with intracellular albumin in the presence of antiserum in these experiments has the properties required of a precursor of plasma albumin. The presence in liver extracts of very highly radioactive protein(s) closely akin to albumin, observed by Schreiber *et al.* (1969), Rotermund *et al.* (1970) and by ourselves (Judah & Nicholls, 1971*a*), is confirmed by the independent method of isoelectric fractionation. We have also confirmed *in vivo* our results with slices (Judah & Nicholls, 1971*b*) that the conversion of a precursor polypeptide into albumin is the rate-limiting step in the secretion of newly synthesized plasma albumin. Our results are in marked contrast with those of Glaumann (1970) and of Peters *et al.* (1971), which show very rapid labelling of intracellular albumin. We conclude that their results are vitiated by a failure to obtain radiochemically pure albumin.

Our evidence suggests that no fraction of the intracellular albumin can be a major precursor of plasma albumin, or if it is, its dwelling time in the liver is so short that it cannot be identified by the present method of separation of intracellular structures. In double-label experiments ^{14}C/^{3}H ratio values for intracellular albumin are without exception markedly lower than those of the presumed precursor. However, we also must note two additional possibilities for these results. The first is that liver cells may be divided into those that secrete and those that do not secrete albumin. The second is that newly synthesized albumin is lost from subcellular sites of formation and randomly distributed among subcellular fractions during the course of our fractionation procedure.

We favour the mechanisms by which the precursor is converted into serum albumin at the point of

secretion. The mechanism of albumin transport within the cell would be simplified, since conversion of precursor would set up concentration gradients favouring a flow of precursor towards the secretory sites. Further, this coupling of secretion with conversion would eliminate the problem of secretion of serum albumin against an apparent concentration gradient. In any event we suggest that there is insufficient evidence to compel one to support the hitherto accepted view that albumin synthesized at the ribosomes is transported through channels of the endoplasmic reticulum to the Golgi apparatus for secretion.

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