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The Separation of Intracellular Serum Albumin from Rat Liver

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1. Antibody precipitation of serum albumin from rat liver extracts yields impure preparations of the protein. 2. When rat liver is labelled with L-[1-^{14}C]leucine, antibody precipitation of albumin leads to material that is contaminated with a protein or proteins of very high specific radioactivity. Only 10–25% of the radioactivity of the antibody precipitate is associated with serum albumin. 3. A chromatographic procedure is described that can be used to separate radiochemically pure serum albumin from antibody precipitates obtained from extracts of rat liver. 4. Extracellular albumin secreted by liver slices yields a precipitate with antibody which contains much less radioactive impurity. About 70–90% of the radioactivity is associated with serum albumin. Serum albumin separated by antibody precipitation from rat serum labelled *in vivo* was not contaminated with the radiochemical impurities associated with intracellular albumin. 5. A simple method is described of obtaining the content of serum albumin in rat liver extracts by the technique of isotope dilution and ion-exchange chromatography.

The separation of radiochemically pure serum albumin, from rat liver or from slices cut from rat liver, by antibody precipitation has long been a problem (Campbell & Stone, 1957; Peters, 1962*a*, *b*), since it is recognized that the antibody precipitate might well absorb highly radioactive materials. Gordon & Humphrey (1961) have shown that relatively pure serum albumin may be obtained from rat liver by antibody precipitation followed by solution of the precipitated albumin in acid–ethanol. However, recent work (Schreiber, Rotermund, Maeno, Weigand & Lesch, 1969) has shown that antibody precipitation is inadequate to separate radiochemically pure albumin from rat liver, and that very large errors will occur if the protein precipitate is not further purified by ion-exchange chromatography. In this paper we describe a simple method for the separation of relatively pure serum albumin from antibody precipitates.

MATERIALS AND METHODS

Animals, liver slices and preparation of serum albumin and of antibodies to serum albumin were as described previously (Judah & Nicholls, 1970).

Determination of protein. This was done by measurement of E_{280} for pure or partially purified preparations. Otherwise, the method of Lowry, Rosebrough, Farr & Randall (1951) was used. The reference sample was rat serum albumin.

Radioactivity determination. This was done by scintillation spectrometry and the d.p.m. values were corrected for quenching by using internal standards and automatic external standardization (Bray, 1960; Gordon & Wolfe, 1960).

Radioactive materials. L-[1-^{14}C]Leucine and L-[4, 5-^{3}H]-leucine were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Preparation of radioactive serum albumin. Rats (140g body wt.) were injected intraperitoneally with 200μCi of [^{3}H]leucine or 40μCi of [^{14}C]leucine and killed 90min later.

Albumin was separated by the method of Debro, Tarver & Korner (1957) from rat serum and further purified as necessary as described below. In general, this method gives serum albumin that is essentially 'pure' (see Judah & Nicholls, 1970) but large-scale preparations are sometimes contaminated with traces of α-globulin, which may be detected by immunoelectrophoresis or electrophoresis on polyacrylamide gel.

Electrophoresis on polyacrylamide gel. This was done as described by Tombs (1968) at pH8.9 in the resolving gel with 375mM-tris–HCl. Elution of products from the gel was done as described by Koen & Shaw (1964).

Preparation of liver homogenates. Liver slices, incubated in Ringer solutions were labelled with radioactive leucine (Judah & Nicholls, 1970) and then homogenized in ice-cold 150mM-NaCl–20mM-tris–HCl, pH7.4, so that an approximately 10% (w/v) homogenate was obtained. Then 0.33 vol. of ice-cold 2.6% (w/v) sodium deoxycholate was added, together with 1.0mg of carrier serum albumin (if required), to the homogenate and the suspension centrifuged at 105000$g_{av.}$ for 60min. The clear supernatant was used for the antibody precipitation of serum albumin.

When the preparation was done *in vivo*, a female rat (125g body wt.) was injected intraperitoneally with

30μCi of L-[1-^{14}C]leucine (specific radioactivity 10μCi/μmol). After 15min the rat was killed and the liver removed and chopped coarsely with scissors. It was washed with cold 150mM-NaCl–20mM-tris–HCl, pH7.4, to free it of blood. A 10% (w/v) homogenate was prepared in 150mM-NaCl–20mM-tris–HCl, pH7.4, treated with deoxycholate and the supernatant was prepared as described above.

Antibody precipitation and splitting of specific precipitate. Enough antiserum was added to the deoxycholate extract (about 500mg of original liver) to bring about quantitative precipitation of serum albumin and the mixture was incubated for 60min at 38°C. It was then left overnight in the refrigerator (4–5°C) and centrifuged to collect the precipitate, which was washed three times in 150mM-NaCl. The precipitate was then suspended in 0.6ml of 1% (w/v) trichloroacetic acid and to this was added 2.4ml of 1% trichloroacetic acid in ethanol. The mixture was then cooled in ice and left for 4h at 0°C.

During this time, a precipitate separated, and 4.0ml of 1% (w/v) trichloroacetic acid in ethanol was then added. If desired, carrier serum albumin (5–10mg) could be added in this ethanolic solution. The mixture was centrifuged (1500*g*, 10min) and the clear supernatant collected. Serum albumin was precipitated from it by addition of 2vol. of diethyl ether. The precipitate was collected by centrifugation, the tubes were well drained and the residue was dissolved (as specified below) for ion-exchange chromatography. At this point, a small flocculent precipitate often appeared, consisting presumably of denatured protein. It was removed by centrifugation. This solution was the starting material for all our subsequent operations.

The volumes of reagents used can be varied from the above, but the proportions must be kept constant.

Ion-exchange chromatography. This was done with two systems. (*a*) DEAE-cellulose (DE 52 obtained from W. and R. Balston, Maidstone, Kent, U.K.) was pre-washed with 0.5M-HCl, with removal of fines. It was then washed with 0.5M-tris base, any fines were removed, and the supernatant was replaced with water. The pH was adjusted to about 7.7 with dil. HCl and the cellulose was allowed to settle. The supernatant was removed and replaced with 100mM-tris–HCl, pH7.73. The cellulose was then poured into columns (1cm×50cm) and equilibrated with 100mM-tris–HCl, pH7.73. Samples of protein (5–10mg) were dissolved in 2.0ml of 100mM-tris–HCl, pH7.73, and pumped on to the column by means of a Buchler peristaltic pump (Buchler Instruments, Fort Lee, N.J., U.S.A.) at a rate of about 0.3ml/min. A 150ml linear gradient of 100–300mM-tris–HCl, pH7.73, was used. An L.K.B. series 7000 fraction collector was used to collect fractions (2.5ml) and the protein was monitored by means of a Vitratron optical scanner (Fisons Scientific Instruments, Loughborough, Leics., U.K.) at 280nm. The protein was then collected from the tubes by precipitation with trichloroacetic acid [7% (w/v) final concentration], drained, dissolved in 0.5ml of 1.0M-NaOH and transferred to counting vials. Carrier protein (2–3mg) was added before acid precipitation when it was known that little or no protein was to be expected in a given fraction.

(*b*) CM-cellulose (CM 52 from W. and R. Balston) was pre-washed with 0.5M-tris, fines were removed and the cellulose was washed with water; 0.5M-HCl was then applied. The cellulose was washed with water and suspended in 30mM-ammonium acetate, pH5.50–5.55 (by glass electrode). It is essential to keep to these limits of pH and careful standardization is necessary. The column dimensions, flow rates and equipment were identical with those used for the DEAE-cellulose columns. Samples of protein (5–10mg) were dissolved in 2.0ml of 30mM-ammonium acetate and pumped on to the columns. The pH of the protein sample dissolved in 30mM-ammonium acetate could be varied from pH5.1 to pH5.50 without affecting the results. Ammonium acetate was standardized by the Nessler reagent against a standard sample of ammonium chloride. Details of the elution appear in the text.

Concentration of albumin from eluates. This is done conveniently by acidification with 5–10% (w/v) trichloroacetic acid. The protein precipitate is collected, dissolved in 1% (w/v) ethanolic trichloroacetic acid and the protein precipitated by the addition of 2 vol. of ether. The precipitate is collected by centrifugation and dissolved as desired.

RESULTS AND DISCUSSION

Impurity of albumin separated from antibody precipitates. Rat liver slices (18 slices, about 2g wet wt.) were incubated in two beakers (5ml of Ringer's solution/slice) for 60min at 31°C in an atmosphere of oxygen. Then 10μCi of L-[1-^{14}C]leucine (specific radioactivity 2μCi/μmol) was added to each of the beakers. At 50min later, the slices were removed, homogenized and treated with deoxycholate as described above. The albumin fraction separated from the albumin–antibody precipitate was chromatographed on DEAE-cellulose, by using a 150ml linear gradient of 100–300mM-tris–HCl, pH7.73. Fig. 1 shows that the greater part of the ^{14}C label separates from the u.v.-absorbing material.

The extracellular albumin fraction secreted by the slices into Ringer's solution was also examined. Of the radioactivity of the crude antibody precipitate 75–100% was associated with the purified albumin, in contrast with the findings with liver extracts.

It was plain that a simple antibody precipitation from slice extracts yielded a mixture of radioactive proteins, 80–90% of the radioactivity residing in fraction(s) distinct from serum albumin. Examination of Fig. 1 shows that it is difficult to tell whether any of the radioactivity was truly associated with serum albumin, and we decided to use CM-cellulose chromatography at a substantially lower pH value in the hope that serum albumin would separate better from the radioactive contaminants.

Separation of ^{14}C-labelled serum albumin from homogenates. After some preliminary experiments, labelled intracellular albumin was precipitated with antiserum as described in the preceding section and subjected to chromatography on CM-cellulose in a single buffer (30mM-ammonium

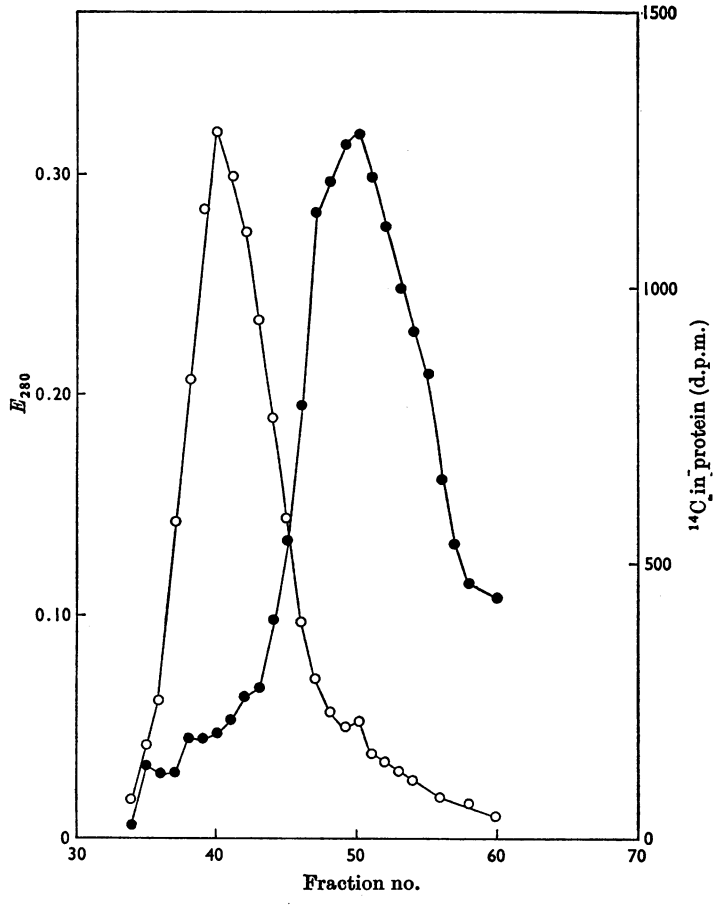

Fig. 1. Separation of intracellular albumin by ion-exchange chromatography on DEAE-cellulose. The albumin was obtained from nine liver slices (200 mg of protein) incubated in Ringer's solution containing L-[1-^{14}C]leucine (specific radioactivity 6 μCi/μmol) for 50 min. The slices were homogenized and the serum albumin was precipitated with antibody as described in the Materials and Methods section. The soluble protein from the precipitate was applied to a column (50 cm × 1 cm) in 2.0 ml of 100 mM-tris–HCl buffer, pH 7.8 (at 20°C), with 10 mg of carrier albumin. The total radioactivity of the sample was about 13000 d.p.m. The separation was done with a 500 ml linear gradient of 100–300 mM-tris–HCl, pH 7.80. ●, E_{280}; ○, radioactivity (d.p.m.).

acetate, pH 5.50); ten fractions were collected of 2.4 ml. After this, a 150 ml linear gradient of 30–150 mM-ammonium acetate, pH 5.50, was applied. The protein separated into two major peaks (fractions A and B, with A leading), and a large radioactive component (fraction C) followed but did not coincide with fraction B. The amount of radioactivity actually present in serum albumin varied from 10–25% of that present in the crude antibody precipitate (Fig. 2*a*).

When ^{14}C-labelled albumin, separated from the serum of rats labelled *in vivo*, was subjected to chromatography on CM-cellulose, the two protein fractions (A and B) were again observed and their specific radioactivities were identical and constant, but fraction C did not appear (Fig. 2*b*).

It was concluded that fraction C was an impurity of high specific radioactivity found in liver extracts but not in rat serum and that the antibody procedure was adequate to separate extracellular but not intracellular albumin in relatively pure form. It was also concluded that rat serum albumin could be separated into at least two distinct fractions (A and B) by chromatography on CM-cellulose.

Proof of the purification. Since fraction A could be obtained so easily, we had to establish (1) that it was a representative sample of the serum albumin applied to the CM-cellulose column and (2) that it

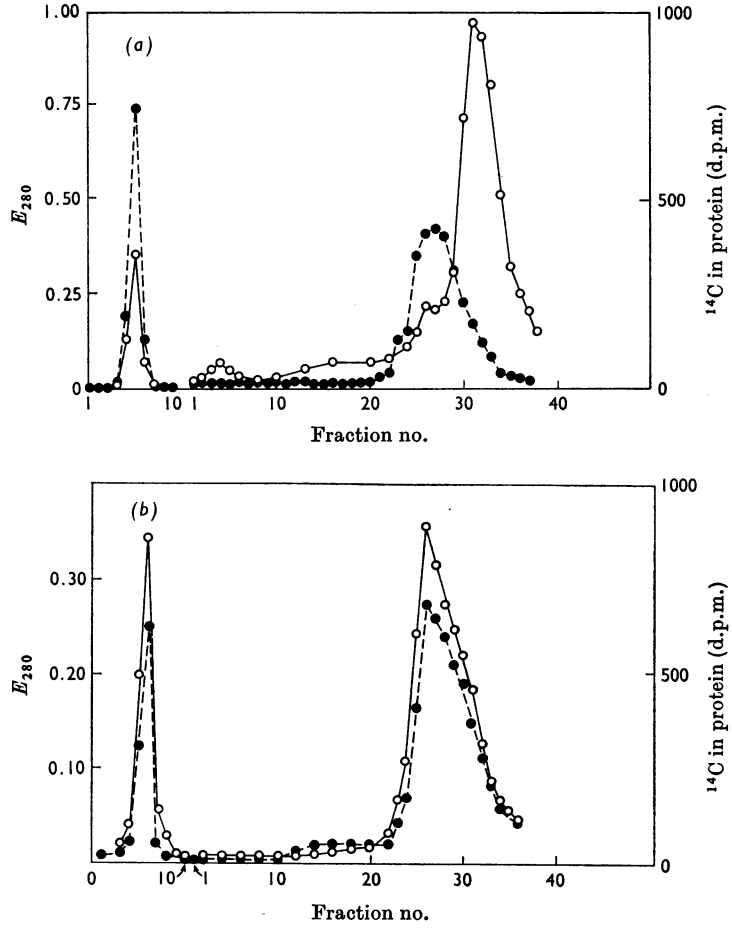

Fig. 2. Chromatography of (*a*) intracellular and (*b*) extracellular albumin on CM-cellulose. The details are as in Fig. 1, except that in (*a*) the sample of albumin was dissolved in 2.0 ml of 30 mM-ammonium acetate, pH 5.50, with 10 mg of carrier albumin (total radioactivity about 7000 d.p.m.) and in (*b*) the sample was 10 mg of rat serum albumin labelled *in vivo* (total radioactivity about 8000 d.p.m.). The chromatography was done by running 30 mM-ammonium acetate for 10 fractions of 2.5 ml each, and then changing to a 150 ml linear gradient of 30–150 mM-ammonium acetate, pH 5.50. ----, E_{280}; ——, radioactivity (d.p.m.).

could be obtained with constant specific radioactivity. This would enable us to study fraction A alone in work on intracellular albumin. We proceeded as follows. The two separate fractions of extracellular serum albumin (A and B) were prepared, collected and concentrated. Electrophoresis on polyacrylamide gel showed that both migrated to the same point on the gel as the untreated original albumin sample, and that no other protein bands were discernible.

Double diffusion of untreated albumin and the two fractions against anti-albumin yielded a pattern of identity. Immunoelectrophoresis of fraction A against antibody to whole rat serum yielded a single precipitin line, corresponding to serum albumin.

Re-chromatography on DEAE-cellulose. Preparations of labelled serum albumin derived by antibody precipitation from homogenates of liver slices were chromatographed on CM-cellulose as described above. The tubes containing fraction A were pooled and concentrated. On chromatography on DEAE-cellulose, the material ran as a single protein peak with constant radioactivity (Table 1, Expt. 1).

Electrophoresis on polyacrylamide gel. An extract was prepared from whole rat liver as described in the Materials and Methods section. Serum albumin was precipitated with antibody, the precipitate split and the soluble proteins were chromatographed on CM-cellulose. The purified albumin samples derived from fraction A from three runs on CM-

Table 1. *Purification of serum albumin*

The albumin was split from the antibody precipitate as described in the Materials and Methods section. Carrier albumin (10mg) was added before chromatography. Expt. 1 was done with rat liver slices. Several runs on CM-cellulose were pooled and rechromatographed on DEAE-cellulose. The specific radioactivity of the protein obtained is the mean ±S.D. of eight tubes. Expt. 2 was done with a rat liver labelled *in vivo*. The results are the means of two separate runs. Expt. 3 was done by the simplified procedure using 5cm columns and two separate runs. The specific radioactivity of the eluted protein is the mean ± S.D. of ten peak tubes. Repetition with a 50cm column gave identical results. The source of labelled albumin was a rat liver labelled *in vivo*. In every case, the albumin sample was that obtained from the first peak (fraction A).

Expt. no.	Fraction	Radioactivity (c.p.m./mg of carrier albumin)	
		Intracellular albumin	Extracellular albumin
1	Released from antibody precipitate	1615	2700
	After chromatography on CM-cellulose	323	2120
	Further chromatography on DEAE-cellulose	356 ± 18	1891 ± 41
2	Released from antibody precipitate	12500	—
	After chromatography on CM-cellulose	3300	—
	After electrophoresis on polyacrylamide gel	3650	—
3	Released from antibody precipitate	8000	—
	After chromatography on CM-cellulose	2218 ± 65	—

cellulose were pooled and concentrated and subjected to electrophoresis on polyacrylamide gel. The protein was eluted from the gel and its specific radioactivity was identical with that of the material obtained from the CM-cellulose chromatography (Table 1, Expt. 2). These procedures were repeated with identical results.

Albumin content of the antibody precipitate by isotope dilution. These experiments could mean that antibody precipitation separates relatively pure albumin contaminated by material of high specific radioactivity. Alternatively, the content of albumin in the precipitate might be low and the balance made up of other radioactive protein(s). We therefore precipitated serum albumin from whole rat liver without labelling, and obtained 1.82mg of presumed albumin after acid–ethanol treatment of the antibody precipitate. A portion (0.91mg) of this protein was mixed with 0.26mg of ^{14}C-labelled serum albumin of known specific radioactivity (2840c.p.m./mg of protein). The mixed proteins were chromatographed on CM-cellulose and the specific radioactivity of the purified albumin was determined. The specific radioactivity of the mixed serum albumin was expected to be 640c.p.m./mg if all the protein derived from the antibody precipitate were albumin; the observed value was 665. This means that at least 0.82mg (91%) of the unknown protein was serum albumin, and is in agreement with the observations of Gordon & Humphrey (1961) that their procedure yields essentially pure serum albumin.

It may also be noted that the method of isotope dilution described here is a very useful technique for the determination of serum albumin in rat liver, and is independent of recovery of protein. The best procedure would be to add labelled albumin before antibody precipitation. Alternatively, if one were studying ^{14}C-labelled serum albumin, the marker albumin could be ^{3}H-labelled. These methods have been used successfully by us.

Simplified procedure. Since we were interested in using this method for the study of the effect of K^+ on the secretion of serum albumin by liver slices, it was necessary to shorten the procedure and to make it suitable for routine use. This was done by using 30mM-ammonium acetate buffer, pH5.50, for elution of fraction A from CM-cellulose columns (5cm × 1cm); 10 fractions (1ml) were collected. The bulk of fraction A was found in tubes 5, 6 and 7, from which it could be concentrated if necessary.

Fraction B and the radioactive contaminants were left on the ion exchanger. The process took approx 30min. After this, the residual proteins on the ion-exchanger were washed off with 150mM-ammonium acetate, pH5.50 (about 15ml was required under our conditions) and discarded or stored frozen at −20°C for future use.

The radioactivity of the unknown sample of serum albumin was determined easily from the specific radioactivity of fraction A, since a known amount of carrier serum albumin was added before chromatography (Table 1, Expt. 3).

Preparative use of CM-cellulose. For preparative purposes we have used a column (2.5cm × 50cm) of CM-cellulose. Serum albumin (150mg) prepared by

the method of Debro *et al.* (1957) is dissolved in 20ml of 30mM-ammonium acetate, pH5.50, and applied to the column at a rate of about 0.3ml/min: fractions (6.0ml) are collected. Elution with 30mM-ammonium acetate yields about 30mg of the leading peak (fraction A). This material gives a single precipitin line on immunoelectrophoresis against antiserum to whole rat serum. Electrophoresis on polyacrylamide gel or cellulose acetate shows a single band due to serum albumin. The material remaining on the ion-exchanger may be recovered by the passage of 150mM-ammonium acetate (about 400ml). It is then concentrated, redissolved in 30mM-ammonium acetate, pH5.50, and reprocessed.

This method is only applicable to serum, and was used to supply samples of serum albumin of sufficient purity for use as antigen.

Conclusion. Antibody precipitation of serum albumin, followed by acid–ethanol extraction of the precipitate yields reliable results with extracellular albumin.

It is unreliable when intracellular albumin is examined in radioisotopic studies of albumin synthesis, since the precipitate is contaminated with radiochemical impurities of high specific radioactivity.

Although there is reason to believe that the procedure of Gordon & Humphrey (1961) gives good results if one is interested in the content of albumin within liver cells, it is prudent to remember that the albumin so separated might be contaminated with significant amounts of other protein.

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