Evidence for the Coupling of Biosynthesis and Secretion of Serum Albumin in the Rat

THE EFFECT OF COLCHICINE ON ALBUMIN PRODUCTION

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1. By using isotopic-dilution techniques it was found that colchicine causes a slight increase in the proalbumin content of liver, from 0.63 ± 0.06 to 0.83 ± 0.10 mg/g of liver, but has no effect on albumin content (0.50 ± 0.05 mg/g of liver). All the proalbumin and 67% of the albumin is found in vesicles from which they are liberated by detergents. 2. Colchicine inhibits secretion of albumin, decreases the rate of conversion of proalbumin into albumin and decreases the rate of incorporation of L-[1- 14 C]leucine into proalbumin. 3. Balance studies *in vivo* show that all the 14 C appearing in serum albumin can be accounted for by the flow of 14 C through the proalbumin, in the presence or absence of colchicine. 4. When cycloheximide is given to the rats, 2 min after [14 C]leucine, further synthesis of protein stops. The label in proalbumin disappears and the proalbumin content of the liver falls, so as to account for the albumin appearing in the plasma. This occurs both in the presence and in the absence of colchicine. By contrast, there is little change in liver albumin. Studies with isolated perfused livers are in agreement with the above. Lumicolchicine has no effect on any of these systems at doses at which colchicine exerts its action. 5. These results suggest that biosynthesis and conversion of proalbumin into albumin, and secretion of serum albumin are controlled at each step.

In our previous work, we reported that proalbumin is synthesized in liver cells and converted into serum albumin before secretion (Judah & Nicholls, 1971; Geller *et al.*, 1972; Judah *et al.*, 1973). The conversion of proalbumin into albumin takes place by the severance of a hexapeptide from the *N*-terminal end of proalbumin (Russell & Geller, 1973, 1975; Quinn *et al.*, 1975). We hoped to find the intracellular site of conversion of proalbumin, but since the secretion of albumin is rapid (Geller *et al.*, 1972) it was necessary to slow the process down. Colchicine has been reported to decrease the secretion of serum proteins (Le Marchand *et al.*, 1973), presumably by its reaction with microtubules (Wilson *et al.*, 1974).

We have investigated the effect of colchicine on the secretion of serum albumin and on the fate of proalbumin in the perfused liver and in the rat. The experiments failed to reveal the site of conversion of proalbumin in the liver. Instead they show that in the presence of colchicine there is little accumulation of proalbumin and none of albumin despite a marked inhibition of secretion, and suggest that each step in the biosynthesis and secretion of albumin is under control.

Materials and Methods

These were as described previously (Quinn *et al.*, 1975; Judah *et al.*, 1973) with the following exceptions.

Albumin in perfusate samples was assayed by quantitative immunoelectrophoresis (Laurell, 1966). Albumin from plasma was separated by the method of Debro *et al.* (1957).

Purification of proalbumin and liver albumin

The proteins were precipitated from deoxycholate extracts of liver (or of subcellular fractions) with anti-(rat albumin) and recovered as described by Quinn *et al.* (1975). They were separated from each other by chromatography on DEAE-cellulose (Whatman Biochemicals, Maidstone, Kent, U.K.). Protein (about 2 mg) was applied to a column (1 cm $\times$ 10 cm) equilibrated to 40 mM-KH₂PO₄, adjusted to pH 6.2 with KOH. After the sample was washed on to the column with a further 2–5 ml of column buffer, proalbumin was eluted with 60 ml of 60 mM-KH₂PO₄, pH 6.2. Proalbumin began to appear after the passage of 10 ml of this buffer and 90% of the protein appeared in the next 30 ml. The proalbumin fraction appearing between 10 and 30 ml of eluent was concentrated to approx. 1 ml by ultrafiltration. Protein was determined by the method of Lowry *et al.* (1951) and the radioactivity by liquid-scintillation spectrometry as described previously (Judah & Nicholls, 1971).

The hepatic albumin remaining on the column was eluted with 200 mM-KH₂PO₄, pH 6.2, the protein coming off sharply at the front. The specific radio-

activity of the albumin could be determined without concentration since the bulk of the protein was contained in 4 ml of eluent.

Proalbumin and albumin content of liver

This was measured in an isotopic dilution experiment in which 10 μ g of proalbumin (specific radioactivity 2×10^6 d.p.m./mg) was added to 30 ml of 0.25 M-sucrose, containing 0.7% (w/v) deoxycholate. The rat liver (approx. 6 g wet wt.) was homogenized in this mixture, which was centrifuged at $100000g_{av}$ for 60 min. The albumin fractions in the supernatant were precipitated by anti-rat albumin and the specific radioactivity of the proalbumin was determined as described above.

Albumin was determined in the same way, but the liver was perfused free of blood before homogenization so as to remove blood contamination. 3H -labelled rat albumin (0.5 mg; specific radioactivity 360000 d.p.m./mg) was added to the homogenizing medium (see above). The use of ^{14}C and 3H markers allows the simultaneous determination of proalbumin and albumin and provides an automatic test of the separation, which was confirmed as described before (Quinn *et al.*, 1975).

Determination of plasma volume

^{14}C -labelled rat albumin (2.5 mg; specific radioactivity 1500 d.p.m./mg) was injected intravenously in 0.25 ml of 150 mM-NaCl. The rat was bled 5 min later and the ^{14}C content of the plasma determined.

Determination of amino acid pools and leucine specific radioactivity

L-[^{14}C]Leucine [5 μ Ci mixed with 0.9 μ mol of cycloheximide in 150 mM-NaCl (approx. 0.5 ml)] was injected intravenously into a 160 g rat. After 30 s the rat was bled out by severing the jugular vein. At 60 s the liver was homogenized in 20 ml of ice-cold 11.5 M-HClO₄. The protein precipitate was centrifuged off and the supernatant adjusted to pH 6 with 6 M-KOH. KClO₄ was allowed to settle overnight at 2°C and then removed.

The supernatant was treated with 5 ml of 1 M-acetic acid (pH of mixture approx. 3.5), and 2 g of Dowex 50W (X8) resin was added. The mixture was shaken at intervals for approx. 60 min. The supernatant was poured off the resin, which was washed with 3 $\times$ 5 ml portions of water. The amino acids were eluted with 3 $\times$ 1.0 ml portions of 4 M-NH₃ solution. These were combined and evaporated to dryness in a rotary evaporator. The residue was taken up in 0.2 ml of 10 mM-HCl and 0.01 ml was subjected to automatic amino acid analysis.

Lumicolchicine was prepared by the method of Wilson & Friedkin (1966) and monitored by the spectral change.

Liver perfusion

This was done by the method of Hems *et al.* (1966). The perfusate (80 ml) consisted of Krebs-Henseleit NaHCO₃-based saline (Krebs & Henseleit, 1932) containing: (a) 2.5% (w/v) bovine serum albumin [fraction V, Sigma (London) Chemical Co., London S.W.6, U.K.], which was dialysed against water (three changes at 2°C for 48 h) before use; (b) 14 mM-D-glucose; (c) aged human erythrocytes [washed three times in Krebs-Henseleit buffer (as above) to give an haematocrit of 25%]; (d) amino acids obtained as a concentrate (DMEM $\times$ 10; Flow Laboratories, Irvine, Ayrshire, U.K.), which was added to the medium (25 ml/litre).

The gas phase was O₂ + CO₂ (95:5) giving a pH of approx. 7.4. The input pressure was 15 cm head of medium, which gave a flow of approx. 2.5 ml/min per g wet wt. of liver.

Livers were always perfused for approx. 20 min before starting the experiment, to allow the system to settle down.

In experiments requiring a change of medium, an equal volume of perfusate was equilibrated to the gas phase and temperature in a second oxygenator, and circulation changed to this medium when required.

Results

Effect of colchicine on proalbumin and albumin content of rat liver

Colchicine (2.5 mg/kg) caused a decrease in the rate of appearance of labelled albumin in the plasma. The effect was slow to appear, starting 60 min after treatment and becoming near-maximal at 240 min. At 16 h after injection of colchicine, the effect was still the same as at 240 min (Fig. 1). The amount of proalbumin and of albumin in the livers of such rats was therefore determined by isotope dilution of added marker [^{14}C]proalbumin and [3H]albumin to the liver homogenates as described in the Materials and Methods section. It is essential to use this method, because there are large losses (approx. 70%) of proalbumin and albumin from deoxycholate-treated homogenates.

The control livers (approx. 6 g wet wt.) gave 0.63 ± 0.06 mg of proalbumin/g of liver (five rats) and 0.5 ± 0.05 mg of albumin/g of liver. All the proalbumin and 67% of the albumin was bound in vesicles from which they could be released by detergents; 0.17 ± 0.02 mg of albumin/g of liver was found free in the cytosol.

In colchicine-treated rats, the hepatic albumin content was unchanged, but proalbumin rose to 0.83 ± 0.1 mg/g of liver at 2 h (five animals). At 4 h after colchicine treatment, the values for albumin and proalbumin were the same as at 2 h. When colchicine (1 mg/kg) was given to our rats, the results

Fig. 1. Effect of time of exposure to colchicine on serum albumin secretion in rats

Colchicine (2.5 mg/kg), dissolved in 150 mM NaCl, was given by subcutaneous injection at time zero. At the times shown, each rat was given L-[1-¹⁴C]leucine (5 μ Ci) by intravenous injection. Then 30 min after the label was given, the rats were bled and the [¹⁴C]albumin was determined. Each point represents the mean values of four poisoned as compared with four control rats.

were indistinguishable from those obtained with the higher dose. Since the livers of our rats (6 g wet wt.) secrete 6 mg of albumin/h, one might have expected an accumulation of approx. 10 mg at the end of 4 h. This could only mean that there was a net decrease in the formation of albumin owing to either decreased synthesis or increased breakdown.

Experiments with isolated perfused livers

Fig. 2 shows that colchicine (10 μ M) had a progressive effect on the rate of secretion of serum albumin. The effect was maximal after 60 min. At the end of perfusion (110 min) the control liver had secreted 9.6 mg of albumin, but the colchicine-poisoned liver only 4.7 mg. However, the content of proalbumin and hepatic albumin was the same in both the control and the poisoned livers (proalbumin, 4.0 mg; hepatic albumin, 1.5 mg). The albumin content of the perfused liver is lower than that of livers *in vivo*. We have no explanation for this change in content. This confirms the conclusion that colchicine causes a net decrease in the production of serum albumin.

As colchicine takes a long time to exert its effect, the following experiment was done with livers of rats which had been poisoned with colchicine (2.5 mg/kg) for 2 h before they were perfused. By this means, decreased albumin secretion could be observed

immediately. To separate the effect of colchicine on secretion of albumin from any action on synthesis, the livers were perfused for 20 min to allow them to settle down and then the perfusate was replaced by fresh medium containing cycloheximide (100 μ M).

Fig. 2. Effect of colchicine on secretion of serum albumin by the perfused liver

The livers were perfused and the albumin was assayed as described in the Materials and Methods section. Colchicine (10 μ M) was added at time 0 on the abscissa after a 20 min initial perfusion. The zero-time albumin of the perfusate is about 3 mg, and is mainly due to residual rat plasma in the system. The temperature was 37°C. The gas phase was O₂ + CO₂ (95:5). The results are from one control and one colchicine-treated liver. The experiment was repeated three times with identical results. ○, Control; ●, colchicine-treated.

Fig. 3. Effect of colchicine on secretion of albumin after the addition of cycloheximide

Rats were poisoned with colchicine (2.5 mg/kg) and left for 120 min, after which their livers were perfused for 20 min. The perfusate was then replaced by fresh medium containing cycloheximide (100 μ M). This removed all the rat albumin from the system. All other details are as in Fig. 2. ○, Control; ●, colchicine-treated.

The change of medium removes residual rat albumin from the system. The cycloheximide stops protein synthesis and allows the secretion of pre-formed protein to be observed. Fig. 3 shows that, in the absence of protein synthesis, approx. 2mg of albumin appeared in the perfusate in both control and colchicine-treated livers, but at very different rates. We concluded that colchicine diminished the rate of secretion of serum albumin.

The above experiments show that production of albumin, as well as its secretion, is restricted in the colchicine-poisoned liver. The decreased production of serum albumin could be due to either diminished synthesis or to increased intracellular degradation.

Synthesis of proalbumin and intracellular albumin. The perfused liver may be used for the determination of the rate of synthesis of proalbumin and of albumin, by taking advantage of the method of Mortimore *et al.* (1972). The use of radioactive isotopes in the determination of rates of synthesis of proteins is complicated by intracellular proteolysis, which feeds unlabelled amino acids to the precursor pool. This problem can be circumvented by the addition of high concentrations of the chosen amino acid precursor. Mortimore *et al.* (1972) used 15mM-L-valine together with [^{14}C]valine for this purpose. Table 1 shows that the incorporation of valine into proalbumin and hepatic albumin is decreased to the

same extent by colchicine as is the rate of secretion (cf. Fig. 3).

Experiments on rat liver in vivo

The animals were dosed with colchicine (2.5mg/kg body wt.) by subcutaneous injection, at the same time in the morning for each experiment, and control animals were investigated simultaneously. At 2h after treatment, L-[1- ^{14}C]leucine (5 μCi) was given to each animal by intravenous injection. Figs. 4, 5 and 6 show the progress of the label through proalbumin, hepatic albumin and into serum albumin respectively, in control and colchicine-treated animals. The data show that the rate of loss of proalbumin and the rate of appearance of albumin in the plasma are much diminished in the presence of colchicine. There is an accumulation of label in the hepatic albumin in the colchicine-treated rats (Fig. 5), but, as shown above, there is no measurable increase in the albumin content of these livers. This must therefore mean that a small amount of highly radioactive albumin has been trapped in the liver.

Table 1. Incorporation of [^{14}C]valine into proalbumin, hepatic albumin and liver proteins

The colchicine-treated livers were derived from rats pretreated with colchicine, 2h before perfusion. For Expt. 1, the dose was 2.5mg/kg; for Expts. 2 and 3, it was 1mg/kg. The perfusion was as described in the Materials and Methods section, except that 15mM-L-valine, together with 25 μCi of L-[U- ^{14}C]valine, was added to the perfusate to start the experiment. Then 10min later, the livers were rapidly homogenized in 30ml of ice-cold 150mM-NaCl containing 0.67% (w/v) sodium deoxycholate. The homogenates were centrifuged (100000 g_{av}) for 60min and the supernatant was treated with excess of anti-albumin. The antigens were separated and analysed as described in the Materials and Methods section.

Liver	Expt.	Valine incorporated ($\mu\text{mol}/10\text{min}$) into		
		Proalbumin	Albumin	Liver proteins (minus albumins)
Control	1	0.78	0.1	—
	2	0.67	0.13	1.2
	3	0.51	0.12	1.0
Colchicine-treated	1	0.19	0.02	—
	2	0.19	0.04	1.0
	3	0.26	0.05	0.9

Fig. 4. Effect of colchicine on the specific radioactivity of proalbumin

All rats were given 5 μCi of L-[1- ^{14}C]leucine by intravenous injection and killed at the times shown thereafter. The treated group was given colchicine (2.5mg/kg) 120min before the start of the experiment. Proalbumin was separated from the livers by antibody precipitation and ion-exchange chromatography as described in the Materials and Methods section. Each point is the mean of five rats, except the controls at 45min and 60min which were done in duplicate. The s.d. of means was $\pm 15\%$ for times 6, 12 and 30min for both groups. It was $\pm 25\%$ for times 45 and 60min of the colchicine-treated group. $\circ$, Control; $\bullet$, colchicine-treated.

The total radioactivity flowing through the liver and appearing in the plasma as albumin is shown in Fig. 7 for control and colchicine-treated rats. From these data, it is clear that proalbumin must be the

major source of the serum albumin. Further, we can say that all the ^{14}C in proalbumin appears eventually in the plasma albumin of the colchicine-treated rats. In the controls, on the other hand, some 30% of the label in proalbumin cannot be accounted for and is presumably lost by proteolysis.

Fig. 5. Effect of colchicine on the specific radioactivity of hepatic albumin

The details are as described in Fig. 4. The hepatic albumin was separated by antibody precipitation and ion-exchange chromatography as described in the Materials and Methods section. ○, Control; ●, colchicine-treated.

Fig. 6. Effect of colchicine on the specific radioactivity of albumin in the plasma

The details are all as described in Fig. 4. Blood samples were taken at the times shown, and albumin was separated as described in the Materials and Methods section. Each point is a mean value for five rats, with a S.D. $\pm 10\%$ at each point. ○, Control; ●, colchicine-treated.

Fig. 7. Incorporation of L-[1- ^{14}C]leucine into total proalbumin, hepatic albumin and plasma albumin of control (a) and colchicine-treated (b) rats

The data are derived from specific radioactivities of the three proteins multiplied by the total of each protein present in the liver or in the plasma. The proalbumin and hepatic albumin content of the livers and the plasma volumes were determined as described in the Materials and Methods and the Results sections. ●, Proalbumin; ■, liver albumin; ▲, serum albumin.

These findings are in agreement with those of the experiments with perfused livers given in the previous section.

We conclude that the failure to accumulate any large amount of proalbumin or albumin in the livers of colchicine-treated rats is due to a decrease in the synthesis of proalbumin and in its conversion into albumin. These processes appear to keep pace with the lowered rate of secretion.

Rate of disappearance of proalbumin from the liver. If proalbumin is the precursor of serum albumin, its rate of disappearance from the liver should be equal to the rate of secretion of albumin into the serum. After intravenous injection of cycloheximide (1.5 mg/kg body wt.) the proalbumin content of the liver falls rapidly in normal animals (Table 2). When rats are treated with colchicine the rate of loss is halved.

In parallel experiments, L-[1-¹⁴C]leucine (5 μ Ci) was injected intravenously 2 min before the cycloheximide into groups of four rats, two groups of controls and two groups of colchicine-treated animals. One group of each was killed at 10 min after administration of [¹⁴C]leucine for determination of the specific radioactivity of proalbumin and liver albumin. The remaining control group was killed at 30 min after labelling to obtain blood samples as well as to determine the radioactivity of liver proalbumin and albumin. The remaining colchicine-treated group was killed at 60 min after labelling, for the same purposes.

From the specific radioactivity of the proalbumin in the controls (83000 c.p.m./mg of protein) and the total radioactivity appearing in the plasma (180000 c.p.m.) it can be calculated that 2.2 mg of albumin emerged in the 20 min period of the experiment, or at a rate of 1.1 mg/h per g of liver (see Table 2). The corresponding calculation for the colchicine-treated group yielded a rate of secretion of albumin of 0.5 mg/h per g of liver.

Effect of lumicolchicine. Lumicolchicine is an

isomer of colchicine formed by u.v. irradiation of colchicine (Wilson & Friedkin, 1966). It does not bind to tubulin and is not an antimetabolic agent. However, lumicolchicine shares other actions of colchicine, e.g. inhibition of nucleoside transport (Wilson *et al.*, 1974), and is therefore a useful control. Lumicolchicine had no effect on the production of albumin at a concentration of 10 μ M in the perfused liver, or at a dose of 2.5 mg/kg in the rat.

Incorporation of [¹⁴C]leucine into total liver proteins. In agreement with the results in the perfused liver, the specific radioactivity of total protein of the livers of colchicine-poisoned rats was not different from those of control rats.

Amino acid pools. Quantitative analysis of liver amino acids (as described in the Materials and Methods section) showed that glutamic acid, glycine, alanine, lysine and histidine contents of the liver of the colchicine-poisoned rats were decreased. The specific radioactivity of hepatic leucine was raised to 150% of control. The analyses were done twice with identical results. The incorporation of [¹⁴C]leucine into the proteins of the colchicine-treated rats has not been corrected for this difference in specific radioactivity of the label.

The obvious explanation for this difference in amino acid content is that colchicine may have decreased the extent of intracellular proteolysis in the liver. A test of this idea, by using the pre-labelled perfused liver as described by Mortimore *et al.* (1972), showed no difference in the rate of general proteolysis of livers from normal or from colchicine-treated rats.

Plasma albumin contents in colchicine-treated rats. At 24 h after the administration of colchicine, the albumin content of plasma fell from an initial 25.1 $\pm$ 1 mg/ml (ten rats) to 18.4 $\pm$ 2 mg/ml (eight rats). This was not due to increased degradation as was proved by the unchanged rate of disappearance of [¹⁴C]leucine-labelled albumin from the plasma of the

Table 2. Comparison of rates of disappearance of proalbumin and secretion of serum albumin in rats

Animals treated with colchicine were given 1.0 mg of colchicine/kg at 2 h before the start of the experiment. All were given cycloheximide (1.5 mg/kg) and were killed at the times shown, when the livers were homogenized and assayed for proalbumin by isotopic dilution as described in the Materials and Methods section. The calculated rates of serum albumin secretion were obtained from parallel experiments in which the rats were given 5 μ Ci of L-[1-¹⁴C]leucine 2 min before the cycloheximide. They were killed at 10 min and 30 min after injection of cycloheximide, when the specific radioactivities of proalbumin and the total radioactivity in plasma albumin were determined. Five rats were used for each of the zero time values, and three rats each for the 30 min and 60 min groups. The values for proalbumin are means $\pm$ s.d.

Time (min) ...	Proalbumin found (mg/g of liver)			Rate of loss of proalbumin (mg/h per g of liver)	Calculated rate of albumin secretion (mg/h per g of liver)
	0	30	60		
Control	0.63 $\pm$ 0.06	0.1 $\pm$ 0.01	—	1.06	1.10
Colchicine-treated	0.83 $\pm$ 0.1	—	0.32 $\pm$ 0.03	0.51	0.52

poisoned rats. The plasma volumes of control and treated rats were measured (as described in the Materials and Methods section) and found (five animals) to be approx. 4.3 ml/100g body wt. for both groups.

Discussion

Colchicine decreases the rate of secretion of serum albumin without allowing any great accumulation of intracellular precursor proteins, and causes a significant decrease in the rate of synthesis of proalbumin.

The most economic hypothesis which links these observations is that colchicine causes disaggregation of microtubules by blocking polymerization. This leads to diminished secretion, resulting in an inhibition of conversion of proalbumin into albumin and to a decreased rate of synthesis of proalbumin. In other words, we are seeing the operation of a negative feedback control system operating at the sites of conversion and of biosynthesis of proalbumin. Inhibition of proalbumin synthesis must occur at the same time or a little later than inhibition of secretion, otherwise we would have seen a fall in proalbumin content in the poisoned liver (cf. the work with cycloheximide). The observations that colchicine has no effect on incorporation of amino acids into the mass of liver proteins support the view that its action is limited if not only to proalbumin then to secreted proteins in general. There is a striking similarity between the action of colchicine and the effect of low cell K^+ on the secretion of serum albumin (Judah & Nicholls, 1971). It should be mentioned here that colchicine has no demonstrable effect on the K^+ content of liver (P. R. Dorling, P. S. Quinn & J. D. Judah, unpublished work). This seems to us to strengthen the case for the existence of a feedback control system(s).

Kemper *et al.* (1975) suggest that antimitotic drugs block the conversion of pro-(parathyroid hormone) into the active hormone. In contrast with our findings with proalbumin they report an increased radioactivity in the pro-hormone in the presence of the inhibitors. The meaning of this observation is not clear, since they presented no quantitative data.

Ehrlich *et al.* (1974) report that the biosynthesis of procollagen and its conversion into collagen are inhibited by antimitotic drugs. This might seem to be very similar to our results with albumin. However, it must be remembered that the conversion of procollagen into collagen is an extracellular event, catalysed by procollagen peptidase secreted from fibroblasts as a proenzyme (Bornstein, 1974). The effect of antimitotic agents on this system is likely to be very complicated.

Le Marchand *et al.* (1973) have reported that antimitotic drugs decrease the output of triacylglycerols from perfused livers, and relate this

phenomenon to the lowered rate of secretion of lipoproteins. They also record a greatly decreased output of label in serum proteins from their perfused livers in the presence of antimitotic agents, an observation with which we entirely agree.

No doubt observations of this kind will be reported for a variety of secretory systems in the future. We wish to make the point that in the absence of quantitative data on intracellular forms of the proteins studied, wrong conclusions might well be drawn. In the present work, antibody precipitation of albumin and proalbumin from detergent extracts of rat liver yields about 30–40% of the proteins present. Isotopic-dilution studies were required to establish real values. When an abnormal or poisoned tissue is studied, there is no certainty that the yields will be the same as with normal tissue. Therefore the presentation of recovered radioactivity has little meaning unless the specific radioactivity and total content can be determined.

Finally, as a by-product of this work, we have been able to show that the proalbumin content of rat liver falls at a rate compatible with the secretion of albumin into the serum, and that colchicine diminishes the rate of disappearance of proalbumin to the same extent as it decreases the appearance of albumin in the plasma. This seems to prove that proalbumin is indeed the source of serum albumin.

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