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Studies on the Synthesis of Serum Albumin by the Isolated Microsome Fraction from Rat Liver

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Zamecnik & Keller (1954) were the first to show that when radioactive amino acids are incubated with the microsomal fraction from rat liver, together with the soluble cytoplasm and a source of continuously regenerated adenosine triphosphate, an incorporation of amino acid into microsomal protein occurs. Since then the steps in this process of incorporation have been extensively studied; the precise nature of the radioactive microsomal proteins has remained undefined, although some progress in this direction was made by Simkin & Work (1957) by extracting the microsomes with solutions of varied ionic strength and pH. It is therefore not clear to what extent this incorporation of radioactive amino acids into substances precipitated by cold trichloroacetic acid but insoluble in hot acid represents the synthesis of a complete protein. It was in the hope that an answer to this question might be found that the present experiments were initiated.

Experiments on the perfused rat liver indicate that this organ is able to synthesize serum albumin (Miller, Bly, Watson & Bale, 1951). Moreover, it is possible to demonstrate the synthesis of serum albumin in incubated slices of either chick liver (Peters & Anfinsen, 1950) or rat liver (Campbell & Stone, 1957a). This demonstration depended on the use of specific antisera by which means the net synthesis of serum albumin during the incubation period could be shown. Further, if the slices were incubated in the presence of a radioactive amino acid, the serum albumin became radioactive and the degree of incorporation of radioactivity was approximately correlated with the amount of

protein synthesized (Campbell & Stone, 1957a). Peters (1957) later showed that chick-liver microsomes contained a small amount of serum albumin which could be solubilized. It was therefore decided in the present experiments to incubate rat-liver microsomes in the presence of radioactive amino acids under the conditions described by Zamecnik & Keller (1954) and to determine whether the amino acids were incorporated into the serum albumin which could be released from the microsomes. It was assumed that it would not be practicable to demonstrate the net synthesis of serum albumin in such preparations.

The major difficulty in conducting these experiments has been to demonstrate unequivocally that the isolated radioactive protein has all the characteristics of serum albumin. As will be shown, immunological, electrophoretic and solubility criteria have been applied in an attempt to demonstrate this. The use of a wide variety of criteria is particularly important for serum albumin since this protein retains its immunological characteristics even though it has been modified, e.g. by acetylation (Marrack & Orlans, 1954), or split into smaller fragments (Lapresle, 1955; Porter, 1957).

Since the amount of albumin which can be released from the liver microsomes is very small, it has been necessary to take advantage of every procedure which would ensure the isolation of albumin with the highest possible radioactivity. It has previously been shown (Hultin & Decken, 1957; Decken & Hultin, 1958; Campbell & Greengard, 1959) that the rat-liver microsome fraction derived from rats after partial hepatectomy is much more

active with respect to incorporation of amino acids than is a similar fraction from normal rats. Most of the experiments have therefore been conducted with microsomes from regenerating liver.

The present experiments show that it is possible to isolate from the microsome fraction of rat liver that has been incubated with radioactive amino acids a protein which is radioactive and which retains all the properties of serum albumin for which it has been tested. The results are consistent with the hypothesis that the isolated microsomal fraction of rat liver is able to synthesize a specific protein. Preliminary reports of some of the results of these experiments have previously appeared (Campbell, Greengard & Kernot, 1958; Campbell & Greengard, 1958; Campbell, 1958b).

While these experiments were in progress similar studies on two other biological systems have also been reported. Thus Schweet, Lamfrom & Allen (1958) have demonstrated the incorporation of radioactive amino acids into haemoglobin by the isolated microsome fraction from rabbit reticulocytes. Bates, Craddock & Simpson (1958) have demonstrated the incorporation of radioactive amino acids into cytochrome *c* during the incubation of rat-liver mitochondria. Recently a net synthesis of this protein by mitochondria isolated from calf heart has been shown (Bates & Simpson, 1959).

MATERIALS AND METHODS

Chemicals. Adenosine triphosphate (dipotassium salt; ATP) was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Solutions of this substance were brought to pH 7.3 (indicator papers) with KOH before use. The barium-silver salt of phosphoenolpyruvic acid was prepared by the method of Ohlmeyer (1951). Solutions of the free acid were prepared shortly before use and adjusted to pH 7.3 (indicator papers) with KOH.

Radioactive amino acids. Uniformly labelled L-[¹⁴C]-leucine (100 μ Ci, 2.29 mg.), L-[¹⁴C]-lysine (100 μ Ci, 1.13 mg.), L-[¹⁴C]-valine (100 μ Ci, 1.46 mg.) and an acid hydrolysate of algal [¹⁴C]protein (100 μ Ci, 2.7 mg.) were obtained from The Radiochemical Centre, Amersham, Bucks.

Animals. Rats were male Wistar albinos highly inbred at this Institute. Partial hepatectomy was performed by the method of Higgins & Anderson (1931).

Preparation of rat-serum albumin. This was prepared from rat serum by a slight modification of the method described for human albumin by Debro, Tarver & Korner (1957). An equal volume of 10% (w/v) trichloroacetic acid was added to the serum, the supernatant after centrifuging was rejected and the precipitate was washed with 5% (w/v) trichloroacetic acid. The precipitate was then suspended in 1% (w/v) trichloroacetic acid in aq. 96% ethanol in a volume equivalent to three times the original volume of serum. After centrifuging, the extract was dialysed against distilled water and freeze-dried.

Chick-serum albumin. This was the sample the preparation of which was previously described (Campbell, 1955).

Antisera against rat- and chick-serum albumin. Rabbits

were injected with alum-precipitated albumin as described by Campbell (1955). The amount of antibody in the rabbit serum was estimated by serial dilution to be about 10 mg./ml. of serum.

Immunological examination. The purified rat-serum albumin and the extracts from the liver microsomes were tested for antigenic heterogeneity by precipitation in agar either in tubes as described by Oudin (1948) or in plates as described by Ouchterlony (1953). The immunoelectrophoretic method of Kohn (1958) employing cellulose acetate strips was also used.

Experiments in the whole animal

Injection. In the studies on normal liver, a rat (150 g. body wt.) was injected intravenously (femoral vein) with 1 ml. of 0.9% NaCl containing 20 μ Ci of algal [¹⁴C]protein hydrolysate. In the studies on regenerating liver, two rats (150 g. body wt.) were subjected to partial hepatectomy and after 48 hr. each rat received by intravenous injection 0.5 ml. of 0.9% NaCl containing 10 μ Ci of algal [¹⁴C]protein hydrolysate.

Fractionation of liver. Both the normal and operated rats were killed 20 min. after the injection. The liver was rapidly removed and homogenized in 2 g. samples in an all-glass Potter homogenizer with 10 ml. of 0.25 M-sucrose. The tissue suspension was filtered through a double layer of gauze and the nuclear, mitochondrial and microsomal fractions were obtained according to the method of Schneider & Hogeboom (1950). The fractions were stored at -15° until required.

Experiments involving the incubation of isolated microsomes

Preparation of microsomes. Regenerating liver was taken from rats 44-48 hr. after partial hepatectomy. Microsomal preparations were made by a method which followed closely that described by Zamecnik & Keller (1954). The tissue was homogenized in 2.5 times its weight of a medium containing phosphate buffer [0.01 M-MgCl₂, 0.02 M-potassium phosphate buffer (pH 7.8), 0.03 M-KHCO₃, 0.025 M-KCl and 0.35 M-sucrose]. The tissue suspension was centrifuged at 12 000 g for 5 min. The supernatant from this centrifuging was then centrifuged in a model L Spinco preparative centrifuge (no. 40 rotor) for 50 min. at 105 000 g. Some of the clear supernatant was siphoned off with a syringe, leaving behind the fat-rich supernatant. The remainder of the supernatant was decanted and rejected. The pellet was suspended, in 0.4 ml. of medium/g. of original liver, by gentle homogenization in a small all-glass Potter homogenizer. Care was taken to keep all preparations ice-cold during these procedures.

Method of incubation. In the small-scale experiments, the incubation mixture contained 0.4 ml. of the microsome suspension, 0.3 ml. of cell sap (i.e. clear supernatant after centrifuging at 105 000 g), 2 μ moles of ATP, labelled amino acid and 10 μ moles of phosphoenolpyruvic acid, in a total volume of 1 ml. In the large-scale experiments the same proportion of reagents was used, but all volumes were multiplied by a factor of 5 and the incubation took place in 50 ml. Erlenmeyer flasks. After incubation for 50 min. under a continuous stream of N₂ + CO₂ (95:5) at 37°, the microsomes were isolated from the incubation mixture by further centrifuging at 105 000 g for 50 min.

Isolation of proteins

Mixed microsomal or cell-sap proteins. The protein was precipitated by the addition of an equal volume of 10% (w/v) trichloroacetic acid. The protein was then treated with hot trichloroacetic acid and organic solvents according to a modified Schneider procedure (Zamecnik, Loftfield, Stephenson & Steele, 1951).

Serum albumin. When serum albumin was to be isolated from soluble fractions the isolation procedures could be applied directly. However, if the serum albumin present in the microsome fractions was being studied, it was necessary first to disintegrate the particles as follows. The particles from approximately 1 g. of liver were suspended in 2 ml. of 0.9% NaCl by homogenizing in a Potter homogenizer and were then treated for 2 min. with ultrasonics. For this purpose an ultrasonic drill (Mullard Ltd., London; type E7680, 16–24 kcyc./sec.) was used with a stainless-steel probe which had the following dimensions: narrow end 0.25 in. external diameter, 2.5 in. long; wide end 0.75 in. external diameter, 2.5 in. long. The particulate suspension was placed in a glass tube (0.5 in. $\times$ 2.5 in.) which was held in an ice bath. The suspension was then frozen and stored at -15° overnight. Before extraction of albumin the suspension was thawed and centrifuged at 105 000 g for 50 min. to remove the microsomal debris.

Precipitation of albumin by antiserum. The albumin in the clear extracts was isolated as an antibody–antigen complex by the addition of a specific antiserum as described by Campbell & Stone (1957*a, b*). Before the rat-albumin antiserum was added the tissue extracts were ‘cleared’ by the addition of chick albumin, which was removed by the addition of chick-albumin antiserum as previously described.

Isolation of albumin involving electrophoresis. To the tissue extracts was added 2 mg. of rat albumin in 0.9% NaCl. The total proteins were then precipitated by the addition of an equal volume of 10% (w/v) trichloroacetic acid, washed with 5% (w/v) trichloroacetic acid (1 ml.) and the precipitate was treated with 2 ml. of 1% (w/v) trichloroacetic acid in aq. 96% ethanol. This suspension was centrifuged and the residue again extracted with 1% (w/v) trichloroacetic acid in aq. 96% ethanol (1 ml.). The supernatants were combined and dialysed against distilled water. To ensure that the final volume of the residue should be as small as possible, the minimum length of narrow-bore dialysis tubing was used. The precipitate that formed on dialysis was then removed by centrifuging and the clear supernatant (about 5 ml.) was freeze-dried from a 25 ml. round-bottom flask with a B24 standard ground-glass joint. The male joint on the freeze-drier was covered with a piece of lens tissue to collect any of the dried protein that escaped from the flask.

In order to isolate the albumin from the freeze-dried protein, the electrophoresis method of Kohn (1957) was adopted. The protein (1.0–1.5 mg.) was dissolved in 20 μ l. of veronal buffer (1.1%, w/v, sodium barbitone and 0.15%, diethylbarbituric acid, pH 8.6; 1.0–0.5) containing a few crystals of bromophenol blue and was applied with a micropipette to a strip of cellulose acetate paper (20 cm. $\times$ 5 cm.; obtainable from Oxo Ltd., Oxoid Division, Thames House, London, E.C. 4) in a straight line approximately 6 cm. from the cathode end. A sample (0.5 mg.) of a rat-serum albumin preparation obtained by fractionation of

rat serum with Na_2SO_4 was run on a parallel marker strip. A current of 2.6 ma (150 v) was applied for 5–7 hr. The albumin band migrated at about 1 cm./hr. It was usually possible after this time to distinguish clearly the position of the albumin band by the light blue of the complex between albumin and bromophenol blue. If this was not so, the position of the albumin band on the experimental strip was estimated from the relative position on the marker strip. After the completion of electrophoresis the marker strip was dried in an oven at 90° for 5 min. and was then stained with 0.1% (w/v) azocarmine in a solution containing 0.45 M-acetic acid and 0.045 M-sodium acetate. After washing the excess of dye from the marker strip with 10% (w/v) acetic acid, the position of the albumin band could again be checked. Having by this means determined the relative position of the albumin on the experimental strip, a section of the appropriate width was cut out and the paper placed in 5 ml. of 0.9% saline. The remainder of the strip was then stained with azocarmine to check the location of any other protein components.

The strip of cellulose acetate paper containing the albumin was then finely cut with scissors and finally disintegrated in a small Potter-type homogenizer. The suspension was cleared by centrifuging.

Measurement of radioactivity

Mixed proteins. Proteins precipitated with trichloroacetic acid were compressed on to 0.28 cm.² Perspex disks and counted at infinite thickness with a thin mica end-window (2 mg./cm.²) Geiger–Müller counter. For details of the counting procedure and accuracy, see Campbell & Greengard (1959).

Albumin. The method was very similar to that previously described by Campbell & Stone (1957*a*). The albumin-antibody precipitates were dissolved in 0.4 ml. of aq. 5N- NH_3 solution and the solution was transferred to 2 cm.² polythene disks and dried in a vacuum desiccator. When dry, the disks could be counted at infinite thickness and the amount of protein on the disks determined afterwards by dissolving the protein in 0.1N-NaOH and measuring the extinction of the solution at 280 μ . The disks were either counted under the mica end-window counter, as in (*a*) above, or in a Tracerlab SC-16 windowless flow counter.

Homogeneity of rat-serum albumin preparations

The rat-serum albumin used for the preparation of antiserum was prepared by the ethanol–trichloroacetic acid method of Debro *et al.* (1957). For the evaluation of the metabolic experiments it was essential to determine the homogeneity of the albumin preparations. This was done as follows.

Electrophoresis. Compared with either human- or rabbit-serum proteins, it is difficult to separate the α_1 -globulin and albumin components of rat-serum proteins by electrophoresis on paper. However, as shown by Campbell, Kernot & Roitt (1959), this separation may be achieved by the use of cellulose acetate paper, following the method of Kohn (1957). With this method it could be shown that the rat-serum albumin preparation was not contaminated by any other known serum protein.

Immunological methods. When the serum albumin and its antiserum were allowed to diffuse in agar gel in tubes, following the method of Oudin (1948), no evidence of heterogeneity could be detected. Concentrations of antigen

ranging from 10.0 to 0.1 mg./ml. were used. The agar-diffusion method of Ouchterlony (1953), in which diffusion takes place in Petri dishes, was also used. The antiserum to rat-serum albumin was placed in the centre well and rat albumin and whole rat serum were placed in alternate outer wells. Only one precipitation line appeared opposite each well and all the lines merged completely. This indicated that the antiserum reacted only with the albumin component of the whole serum. When the specificity of the antiserum to chick-serum albumin was studied by the Ouchterlony technique no evidence of any cross-reaction of the antiserum with rat albumin could be found.

Preparative electrophoresis on cellulose acetate paper

Resolution of serum proteins. As was previously found (Campbell *et al.* 1959), the α_1 -globulin contained in the crude rat-serum albumin preparation which was used for the marker strip was always well separated from the albumin band. Care was taken not to cut too wide a strip from the albumin band on the experimental paper. In fact, when the remaining paper was stained with azo-carmines some albumin was invariably found on either side of the strip which had been removed.

Amount of protein treated. The major disadvantage of the use of cellulose acetate paper is the low absorptive capacity of the paper for water. Thus with the size of paper used in the present experiments not more than 20 μ l. of solution could be absorbed on the strip, which meant that only a proportion of the freeze-dried protein could be transferred to the paper. In practice this usually amounted to 0.5–1.0 mg., which was the maximum amount of protein that could be fractionated satisfactorily.

Recovery of protein from the paper. A known amount of rat-serum albumin was placed on the paper and, after application of the current for 7 hr., the albumin band was cut out. As explained above, a small amount of albumin was left on the paper around the portion which was removed. The paper was treated as described above and the amount of albumin determined by use of an antiserum (Campbell & Stone, 1957*a*). The amount of albumin in the standard solution applied to the paper was similarly determined. It was thus shown that approximately 60% of the albumin placed on the paper was recovered.

Release of albumin from microsomes by ultrasonic disintegration

In order to determine the extent of the release of serum albumin from liver microsomes the microsomal fraction

was isolated from normal rat liver. Microsome fractions from 1 g. of liver were dispersed in a small Potter homogenizer in 2 ml. of 0.9% NaCl. In one case the suspension was then cleared by ultracentrifuging and in the other the suspension was subjected to ultrasonic treatment and then cleared by ultracentrifuging. The amount of rat-serum albumin in the two supernatants was then determined after treating each with chick-serum albumin and its antiserum. The results of the estimations showed that the rat-serum albumin content of the supernatants was increased from 103 to 147 μ g. of albumin/ml. by the ultrasonic treatment.

RESULTS

Incorporation of amino acids into microsomal proteins in vivo

In order to interpret the results of the studies with isolated microsomes it was considered essential to study the incorporation of amino acids under conditions in which protein synthesis was known to occur, i.e. in the whole animal. Accordingly both normal rats and animals which had been subjected to partial hepatectomy were injected with a mixture of radioactive amino acids. The rats were killed at a time after injection when it was expected that the radioactivity of the total protein of the microsome fraction would exceed that of the other subcellular fractions.

The specific activity of the protein of the various subcellular fractions is given in Table 1. This shows that for both types of liver the microsomal protein

Table 1. *Radioactivity of the protein of the subcellular fractions of rat liver 20 min. after the intravenous injection of [14 C]amino acids*

Radioactivity is expressed as counts/min. at infinite thickness/0.28 cm.² with the mica end-window counter. The results obtained with the two types of liver are not comparable.

Fraction	Normal liver	Regenerating liver
Nuclear	102	72
Mitochondria	119	87
Microsome	413	177
Cell sap	78	65

Table 2. *Radioactivity of the rat-serum albumin present in the supernatants of the disintegrated microsomes obtained from the liver of rats 20 min. after injection of [14 C]amino acids*

The microsome pellets were homogenized, treated with ultrasonics, frozen and the debris was removed. Chick-serum albumin (60 μ g.) was added to the supernatants and was then removed by the addition of antiserum. Rat albumin was then precipitated from the supernatants by the addition of specific antiserum. The radioactivity of the antibody precipitates was determined at infinite thickness with the windowless flow counter. Specific activity is expressed as counts/min./mg. of albumin. The results obtained with the two types of liver are not comparable.

	Normal liver		Regenerating liver	
	Counts	Specific activity	Counts	Specific activity
Chick albumin	216	3 600	128	2130
Rat albumin	599	13 450	382	3638

had a higher radioactivity than that of the other fractions.

Table 2 shows the radioactivity of the rat-serum albumin in the supernatants of the disintegrated microsomes. The amount of serum albumin released from the microsome pellet obtained from approximately 1 g. of liver was 78 μ g. for normal liver and 22 μ g. for regenerating liver. In order to check that the radioactivity of the precipitate of rat albumin was not due to the non-specific adsorption of other radioactive substances, chick albumin was first added to the extracts and was then removed by the addition of an antiserum to chick albumin (Campbell & Stone, 1957*a, b*). It will be seen that the chick albumin precipitates had a considerable activity but that this was less than that of the rat-albumin precipitates. In another experiment the treatment with chick albumin was carried out twice, the activity of the second precipitate being only one-third of that of the first.

In Table 3 the results of the determination of the radioactivity of the microsomal albumin before and after its isolation by the trichloroacetic acid-ethanol procedure are given. The fact that the isolation procedure increases the specific activity of the serum albumin suggests that the albumin-antiserum precipitates obtained from the original extract were contaminated with substances having an absorption at 280 $m\mu$ with a lower specific radioactivity than that of the rat-serum albumin.

From Table 4 it will be seen that the specific radioactivity of the rat-serum albumin from normal liver is reduced as a result of electrophoresis but that from regenerating liver is not significantly affected. Evidence that the radioactivity remained associated with the albumin even after electrophoresis was obtained by combined radioautography on an X-ray film (Kodirex, Kodak Ltd., London) and immunoelectrophoresis (Kohn, 1958). These methods showed that whereas much of the radioactivity was present in the albumin fraction there was a radioactive band at the point of application of the protein to the paper. This was probably due to denatured albumin.

Table 5 shows a comparison of the radioactivity of the albumin extracted from the microsome fraction, before purification by the trichloroacetic acid-ethanol procedure, with that of the insoluble microsomal proteins. To calculate these results

use was made of the factor previously determined (Campbell & Stone, 1957*a*) for converting counts/min./mg. of protein at infinite thinness into counts/min. at infinite thickness/cm.². It will be seen that the serum albumin is much more radioactive than is the insoluble protein although the difference is much smaller for regenerating liver than for normal liver. In regenerating liver the synthesis of tissue protein might be expected to be relatively faster than in normal liver. Severina (1956) found that whereas the rate of synthesis of serum albumin by slices of regenerating chick liver was depressed below normal 24 hr. after operation it rose above normal after 2 days.

Table 3. *Radioactivity of rat-serum albumin after isolation by trichloroacetic acid-ethanol from supernatants of the disintegrated microsomes from regenerating liver labelled in vivo*

Carrier rat-serum albumin (2 mg.) was added to the supernatant containing about 1.2 mg. of albumin and the radioactivity of the rat-serum albumin was determined on a sample as described in Table 2. The rat-serum albumin was then isolated by the trichloroacetic acid-ethanol procedure (see Methods section), and the activity of this purified albumin was again determined by the use of the antiserum. Radioactivity is expressed as counts/min./mg. of albumin.

	Before isolation	After isolation
Chick albumin	503	—
Rat albumin	1084	1560

Table 4. *Effect of electrophoresis on the radioactivity of rat-serum albumin isolated from the supernatants of disintegrated microsomes from normal and regenerating liver labelled in vivo*

The rat-serum albumin which had been purified by the trichloroacetic acid-ethanol procedure (see Tables 2 and 3) was subjected to electrophoresis on cellulose acetate paper (see Methods section). The albumin was removed from the paper and precipitated by the addition of antiserum. The radioactivity of the precipitate was determined with the windowless flow counter. Results are expressed as counts/min./mg. of albumin.

	Normal liver	Regenerating liver
Before electrophoresis	1375	1560
After electrophoresis	1083	1531

Table 5. *Comparison of radioactivity of albumin isolated from the microsomes and insoluble microsomal protein after intravenous injection of [¹⁴C]amino acids*

Results are expressed as counts/min. at infinite thickness/cm.² with the mica end-window counter.

	Insoluble protein	Albumin	Ratio: Albumin Insoluble protein
Normal liver	970	20 900	22
Regenerating liver	730	5 400	8

Studies on isolated microsomes

In the initial experiments in which the microsome fraction from regenerating rat liver was incubated with radioactive amino acids, a source to regenerate ATP and the cell sap, the methods used for the characterization of the serum albumin were entirely immunological. The results of a typical experiment are shown in Tables 6 and 7. Table 6 shows that the presence of phosphoenolpyruvate had a marked effect on the incorporation of radioactive amino acids both for the microsomal protein and the cell-sap protein. From Table 7 it will be seen that the presence of phosphoenolpyruvate greatly enhanced the specific radioactivity of the microsomal rat albumin. It seems probable that the activity of the latter was not due merely to contamination with other radioactive substances since the chick-albumin precipitates had a much lower radioactivity than that of the rat albumin. However, with the cell-sap proteins there was no significant difference in the activity of the rat albumin in the presence and absence of phosphoenolpyruvate. Moreover, the radioactivity of the chick-albumin precipitates was so high that the apparent activity of the rat-albumin precipitates could well have been due to contamination with radioactive substances other than rat albumin. The activity of the second chick-albumin precipitate was in every case less than that of the first. However, with microsomal albumin in the presence of phosphoenolpyruvate the difference was not significant. In a similar experiment a further treatment with chick albumin was carried out after the precipitation of the rat albumin. Here again the chick-albumin precipitate had a similar activity to that of the other chick-albumin precipitates. Approximately 30 μ g. of rat albumin was obtained from the microsome pellet equivalent to 1 g. of rat liver. Since such a pellet contains

about 5 mg. of protein the albumin represents 0.5–1.0 % of the total microsomal protein.

A similar experiment to that just described was done with microsomes isolated from normal liver, except that [14 C]leucine and [14 C]valine were used. The insoluble protein from the microsomes had a radioactivity of 27 and 562 counts/min. at infinite thickness/0.28 cm.² respectively when the incubation was carried out in the absence and presence of phosphoenolpyruvate. The results of the determination on the radioactivity of the extracted albumin are shown in Table 8. The specific activity of the rat-albumin precipitate in the presence of phosphoenolpyruvate was much greater than in its

Table 6. *Incorporation of [14 C]leucine and [14 C]lysine into microsomal and cell-sap proteins in isolated preparations from regenerating liver*

Microsomes and cell sap (in quantities given in the Methods section) were incubated under $N_2 + CO_2$ (95:5) for 50 min. at 37° with 2 μ moles of ATP, and 2 μ c each of [14 C]leucine and [14 C]lysine with and without 10 μ moles of phosphoenolpyruvate (PEP) in a total volume of 1 ml. The microsomes were isolated after incubation by centrifuging and were ultrasonically disintegrated. The rat albumin was removed from the solubilized proteins by the addition of antiserum. The microsomal protein refers to the insoluble proteins together with the non-albumin soluble protein. The soluble fraction of the incubation mixture containing the cell sap was freed of rat albumin by the addition of antiserum. Cell-sap protein therefore refers to the soluble proteins of this fraction other than albumin. Radioactivity is expressed as counts/min. at infinite thickness/0.28 cm.² in the mica end-window counter.

	Microsomal protein		Cell-sap protein	
	Control	Plus PEP	Control	Plus PEP
Radioactivity	69	738	9	189

Table 7. *Radioactivity of rat-serum albumin isolated from the microsomes and cell-sap protein of regenerating liver after incubation with [14 C]lysine and [14 C]leucine*

The supernatants obtained after treatment of the microsomal pellet with ultrasonics were 'cleared' twice by the addition of chick-serum albumin (60 μ g.) and its subsequent removal with antiserum. The soluble fraction from the incubation mixture was similarly treated. Finally the rat-serum albumin in each fraction was precipitated by the addition of anti-serum to rat albumin. Specific radioactivity is expressed as counts/min./mg. of albumin as determined in the mica end-window counter. PEP, Phosphoenolpyruvate.

	Soluble fraction from disintegrated microsomes				Soluble fraction from incubation mixture			
	Control		Plus PEP		Control		Plus PEP	
	Counts/min.	Specific activity	Counts/min.	Specific activity	Counts/min.	Specific activity	Counts/min.	Specific activity
Chick albumin 1	17	284	29	484	152	2530	178	2970
Chick albumin 2	4	67	28	468	72	1200	127	2120
Rat albumin	9	50	227	7100	145	805	153	845

Table 8. *Radioactivity of rat-serum albumin isolated from normal rat-liver microsomes incubated with [¹⁴C]leucine and [¹⁴C]valine*

The incubation procedure was as described under Table 6 except that all quantities were increased by a factor of 5, as described in the Methods section. To each flask containing 5 ml. of the incubation mixture 5 μ C each of [¹⁴C]leucine and [¹⁴C]valine was added. Rat albumin was obtained from the microsome pellet after incubation by treatment with ultrasonics. Results are expressed as counts/min./mg. of albumin at infinite thinness as determined in the windowless flow counter. PEP, Phosphoenolpyruvate.

	Control		Plus PEP	
	Counts/min.	Specific activity	Counts/min.	Specific activity
Chick albumin 1	14	230	188	3120
Chick albumin 2	7	115	103	1710
Rat albumin	7	500	164	5870

Table 9. *Effect of various isolation procedures on the radioactivity of albumin isolated from microsomes obtained from regenerating rat liver, incubated with [¹⁴C]leucine and [¹⁴C]valine*

The procedure for the incubation was as described in the Methods section under large-scale experiments. Each incubation flask containing 5 ml. of the incubation mixture contained 7.5 μ C each of [¹⁴C]valine and [¹⁴C]leucine, together with phosphoenolpyruvate, ATP and cell sap. Controls without phosphoenolpyruvate were not carried out.

(a) Procedures applied directly to the supernatant from disintegrated microsomes. For details see Table 7.

	Counts/min.	Specific radioactivity
Chick albumin 1	51	850
Chick albumin 2	29	500
Rat albumin	67	5350

(b) Carrier rat-serum albumin (2 mg.) was added to the supernatant from the disintegrated microsomes (containing approx. 200 μ g. of albumin) and the albumin re-extracted by the trichloroacetic acid-ethanol procedure. Some of this albumin was then subjected to electrophoresis. The results are the average of two determinations. In another determination the extracts were 'cleared' with chick albumin before addition of rat-albumin antiserum. The chick-albumin precipitates were not significantly radioactive. Radioactivity was determined with a mica end-window counter. Results are expressed as specific activities of albumin (counts/min./mg.).

	Before electrophoresis	After electrophoresis	Change (%)
Specific activity	325	238	26
Specific activity after clearing	410	273	33

absence. However, compared with regenerating-liver preparations the contamination of the chick albumin is much greater with normal liver microsomes. This may be due to the fact that the amount of radioactive amino acid used for both preparations is similar but that the incorporation of radioactivity is much greater with regenerating-liver preparations.

Several experiments were carried out with larger

amounts of microsomes from regenerating liver in order that sufficient radioactive albumin might be obtained to check the effect on its radioactivity of purification by the trichloroacetic acid-ethanol procedure and electrophoresis. The results of a typical experiment are shown in Table 9. In this experiment the radioactivity of the insoluble protein from the microsomes was 2742 counts/min. at infinite thickness/0.28 cm.². As seen in Table 9 (a), the specific activity of the microsomal rat albumin was very much higher than that of the chick-albumin precipitates, which confirms the previous experiments. It may be observed, Table 9 (b), that the activity of the rat albumin was reduced by electrophoresis. The effect of 'clearing' the extracted protein both before and after electrophoresis was to increase slightly the specific activity of the rat albumin.

In another experiment carried out under exactly the same conditions the results obtained were very close to those just described. The opportunity was taken to determine the effect on the specific radioactivity of the microsomal albumin of the trichloroacetic acid-ethanol step. The activity dropped from 423 to 319 counts/min./mg. of albumin. This is in contrast with the effect of this procedure on the microsomal albumin labelled *in vivo*. In two respects the results in this experiment did differ from those of the previous one. First, the precipitate of the chick albumin used to 'clear' the microsomal albumin isolated by the trichloroacetic acid-ethanol procedure was significantly radioactive. However, the second chick-albumin precipitate was inactive and in no case was there any activity in such precipitates when the rat albumin had first been subjected to electrophoresis. Secondly, the reduction in the specific radioactivity of the rat albumin on electrophoresis was greater than previously, being 46 % instead of 26 %. The effect of clearing with chick albumin on the specific radioactivity of the rat albumin was to reduce the activity of the protein before electrophoresis but not to alter that of the protein after electrophoresis.

Table 10. Comparison of the radioactivity of microsomal albumin and insoluble microsomal protein after incubation of microsomes from both normal liver and regenerating liver with [^{14}C]leucine and [^{14}C]valine

Results are expressed as counts/min. at infinite thickness/cm.² with the mica end-window counter and were calculated from the data in Tables 8 and 9.

	Insoluble protein	Albumin	Ratio: Albumin Insoluble protein
Normal liver	2220	9 170	4.1
Regenerating liver	8800	25 700	2.9

A radioautograph of the cellulose acetate strip used for the electrophoresis of the rat-serum albumin showed that, apart from the radioactivity due to the denatured protein at the point of application, the activity was concentrated around the area from which the albumin had been extracted.

Just as the radioactivity of the microsomal albumin could be compared with that of the insoluble microsomal proteins after intravenous injection of radioactive amino acids (see Table 5), so a similar calculation can be made for the experiments *in vitro*. The results of such a calculation are shown in Table 10.

In both these and the earlier experiments the rat albumin in the medium after incubation of the microsomes appeared to have some radioactivity when precipitated by addition of rat-albumin antiserum. However, after addition of carrier rat albumin to the extracts and isolation by the trichloroacetic acid-ethanol method the activity was always low and sometimes the rat albumin was inactive. Electrophoresis removed any remaining activity in these samples of rat albumin. Thus in no case was rat albumin which retained its radioactivity after electrophoresis isolated from the incubation mixture.

DISCUSSION

It must be accepted that the demonstration of a net synthesis is the best criterion of the synthesis of a complex substance such as serum albumin under conditions *in vitro*. In liver slices such a net synthesis of serum albumin during the period of incubation has been demonstrated but at least two considerations made it unlikely that such a criterion could be successfully applied to a cell-free system. First, studies with radioactive amino acids have indicated that, compared with slices, the protein-synthetic activity of isolated liver microsomes is comparatively low and rapidly declines on incubation at 37°, and, secondly, incorporation of radioactive amino acids, and hence protein synthesis, depends on the incubation of the liver microsomes in the presence of cell sap which contains serum albumin. Attempts to reduce the

amount of serum albumin in the incubation mixture, by substituting whole cell sap by pH 5.0 fraction (Keller & Zamecnik, 1956), failed because precipitation at pH 5.0 did not effect any significant degree of fractionation as between cell-sap proteins and serum albumin. Because of these difficulties it has been necessary to use the incorporation of radioactive amino acids into serum albumin as the criterion of protein synthesis. Although it follows from definition that proteins are not synthesized in the absence of incorporation of amino acids, the mere demonstration of such incorporation cannot be taken as unequivocal evidence of protein synthesis *de novo*. The reasons for this conclusion have already been discussed at length (e.g. Campbell, 1958a), so that it will suffice here to confine the discussion to matters particularly relevant to the present problem.

Whereas for many years the microsome fraction remained only as a cytoplasmic concept a much better understanding of the morphological structure of this fraction, at least for rat liver, has arisen from the work of Palade & Siekevitz (1956), who have shown that the rat-liver microsome fraction corresponds mainly to the rough endoplasmic reticulum of the cytoplasm. In view of this it was not surprising that Peters (1957) should find some serum albumin in the soluble protein obtained from the chick-liver microsomes by treatment with deoxycholate. Peters showed that when chick-liver slices were incubated under conditions in which there was evidence of protein synthesis albumin was released from the microsomes.

The results of the present experiments with isolated microsomes, in which the rat-serum albumin was initially only characterized by immunological methods, show that the incorporation of radioactive amino acids was dependent on the presence of a source of continuously regenerated adenosine triphosphate (Table 7). It was necessary to show that this was so because of the amino acid-protein interactions demonstrated by Cornwell & Luck (1958). The use of chick albumin, and its subsequent removal by its antiserum, to check the specificity of precipitation of rat-serum albumin by its antiserum has been studied previously (Campbell & Stone, 1957a, b). A similar method has also

been used by Peters (1957). With microsomal albumin the radioactivity of the chick-albumin precipitates was small compared with that of the rat-albumin precipitates and it is unlikely that the radioactivity of the rat-albumin precipitates was due to the co-precipitation of other radioactive substances. However, the fact remains that the chick-albumin precipitates were radioactive and remained so irrespective of the number of times the process was repeated. The immunological studies appeared to exclude the possibility that the radioactivity of the chick-albumin precipitates was due to a cross-reaction between the chick antiserum and the rat albumin. To determine whether the co-precipitated substances were nucleic acid in nature, the absorption of the dissolved precipitates was determined at both 260 and 280 $m\mu$. However, no evidence of any nucleotides was found so that the reason for the persistent radioactivity of the chick-albumin precipitates remains obscure.

Although these results on microsomal albumin were encouraging it was felt that dependence on a single criterion for the characterization of the radioactive albumin was unsatisfactory. Moreover, Lapresle (1955) and Porter (1957) have shown that fragments of the albumin molecule retain at least some of the immunological properties of the native albumin. The method of Debro *et al.* (1957), in which serum albumin is isolated from an ethanolic extract of a trichloroacetic acid precipitate, has proved to be an extremely useful procedure for the isolation of albumin. When this method is applied to serum proteins there is much evidence that the protein that is obtained consists almost entirely of albumin. However, when the method is applied to the cell-sap proteins subsequent electrophoresis indicates that proteins other than albumin are usually present, although with microsomal extracts the method appears to yield uncontaminated albumin. Therefore, at least for microsomal protein, the specificity of the trichloroacetic acid-ethanol method is such that it alone provides a good test of the purity of the extracted albumin, so that the retention of radioactivity in the albumin isolated from the incubated microsomes by this method amply confirmed the preliminary results.

The final criterion applied to test the purity of the microsomal albumin was electrophoresis. The use of cellulose acetate for the preparative electrophoresis of submilligram amounts of protein has been fully justified. In the experiments *in vitro* the radioactivity of the albumin was reduced as a result of electrophoresis. The percentage reduction in activity (26 and 46% respectively in two experiments) was greater than that found with albumin in which the incorporation of radioactive amino acids had taken place *in vivo*, but there

could be no doubt that the albumin remained significantly radioactive after electrophoresis.

When the rat albumin which had been purified by electrophoresis was treated with chick albumin and its specific antiserum the precipitate formed was without significant radioactivity. This is good evidence that the radioactivity of the microsomal rat albumin was not due to contamination and also demonstrates the value of the electrophoresis procedure.

The comparison which has been made between the results obtained *in vivo* and *in vitro* is a guide to the extent to which the isolated microsomes retain their activity shown *in vivo*. The results *in vivo* both for normal liver and regenerating liver show that the microsomal albumin is much more radioactive than the insoluble or structural protein of the microsomes (Table 5). The difference between the two fractions was much less in regenerating liver than in normal liver, presumably due to the high growth rate of the former relative to the latter. The results *in vitro* (Table 10) do not show much difference between the two types of liver, but again the radioactivity of the microsomal albumin was greater than that of the structural proteins. The difference in the activity of the two fractions was never as great *in vitro* as *in vivo*. This suggests a relatively greater loss in the ability of the isolated microsomes to synthesize albumin as opposed to structural protein.

The experiments with isolated liver microsomes may be said to support the hypothesis that such a tissue preparation is able to synthesize serum albumin. It is, however, difficult to be more precise than this for it cannot as yet be certain that such a preparation is able to assemble all the amino acids required for the synthesis of a complete molecule of serum albumin. The possibility that only a step in this complex process is being studied is a very real one (see, for example, Loftfield, 1958), and further experiments must be designed to determine whether this is so. In this connexion it is a pity that the terminal amino acids in rat albumin have not so far been determined as they have for the serum albumin of many other species (Peters, Logan & Sanford, 1958).

These experiments with the isolated microsome fraction do not in any way exclude the possibility that other organelles of the liver cell are able to synthesize serum albumin. Indeed the demonstration that mitochondria from chick liver (Peters, 1957) and rat liver (Campbell & Greengard, 1958) contain serum albumin makes this protein a most suitable choice for such studies. The experiments of Bates *et al.* (1958) on the synthesis of cytochrome *c* by rat-liver mitochondria are of particular significance in this connexion. Experiments are being carried out to determine the relative contribution

of the various organelles to the synthesis of serum albumin by the liver cell *in vivo*.

It remains to discuss the results of the studies on the incorporation of amino acids into the serum albumin in the supernatant obtained from the medium in which the microsomes were incubated. In the initial experiments, although the rat-albumin precipitates were radioactive there was no evidence of an energy-dependent incorporation (Table 7). The radioactivity of the chick-albumin precipitates was such that the activity of the rat albumin could have been due to coprecipitation of other substances. When the albumin from the incubation mixture was purified by the trichloroacetic acid-ethanol procedure and subjected to electrophoresis no significant radioactivity remained. This again demonstrates the value of these procedures for the removal of contaminating substances. The soluble fraction of the incubation mixture contains approximately twice as much albumin at the end of incubation as can be recovered from the microsomes. Thus it is possible that the failure to detect radioactive albumin in the soluble fraction was due to dilution of the radioactive albumin from the microsomes with the inactive albumin in the soluble fraction. This seems unlikely, however, for in the small-scale experiments the microsomal rat albumin had such a high radioactivity that even if it had been diluted with twice the quantity of inactive albumin it would have remained significantly radioactive. Thus the results of the present experiments suggest that the albumin synthesized in the microsomes during incubation is not released in soluble form.

Simkin (1958) has studied the release of soluble proteins from the microsomal fraction of guinea-pig liver incubated under conditions similar to those of the present experiments. He found that the soluble proteins were released from the microsomes only under conditions in which amino acid incorporation took place. Since it was not possible to uncouple the release from the incorporation it was not clear whether the passage of protein from the microsomes to the soluble fraction was the result of an active process, i.e. secretion, or was merely a displacement of protein from the reticulum due to the formation of new protein. In view of these results involving a mixture of soluble proteins it was a surprise to find that in the present experiments serum albumin was not transferred from the microsomes to the soluble fraction. Since albumin was being synthesized in the microsomes the most likely explanation is that the transfer of albumin from the microsomes to the soluble fraction is an active process and not a displacement. Schweet *et al.* (1958), in their studies on the incorporation of [^{14}C]amino acids into haemoglobin by the microsome fraction from rabbit reticulocytes, isolated

radioactive haemoglobin from the soluble fraction of the incubation mixture. Thus in this case the haemoglobin presumably was released from the microsomes during incubation. The difference between the two systems may be that whereas albumin is synthesized by the liver for 'export', haemoglobin is synthesized by the reticulocyte for its own requirements. Variations in the morphology of the two types of microsomes may also account for the difference.

Since this paper was prepared Jungblut, Heimbürger & Turba (1959) have reported that four proteins with antigenic properties against an anti-serum prepared against purified rat albumin may be extracted from rat-liver microsomes by deoxycholate. Only one of these proteins has the electrophoretic properties of serum albumin. The electrophoretic method described above for the isolation of albumin from the radioactive microsomes clearly separated serum albumin and α_1 -globulin. Hence the conclusions reached in the present paper are not affected by the findings of Jungblut *et al.* (1959).

SUMMARY

1. The microsomal fraction from regenerating rat liver was incubated with radioactive amino acids, adenosine triphosphate, phosphoenolpyruvate and cell sap. The microsomes were separated from the soluble proteins and were disintegrated with ultrasonics. The rat albumin released from the microsomes and in the soluble fraction of the incubation mixture was precipitated by the addition of an antiserum and its radioactivity was determined. The results indicated that there was an incorporation of amino acids into the microsomal albumin but not into the albumin in the medium.

2. The rat albumin isolated from the microsomes and the medium after incubation as described above was purified by the trichloroacetic acid-ethanol method and then subjected to electrophoresis on strips of cellulose acetate paper. The microsomal rat albumin retained its radioactivity throughout the course of these purification procedures.

3. Similar results were obtained with microsomes from normal liver.

4. Identical isolation procedures were applied to albumin isolated from liver microsomes 20 min. after injecting rats with radioactive amino acids. The radioactivity of the rat albumin was determined throughout the course of isolation.

5. A comparison of the radioactivity of the insoluble microsomal protein and the microsomal albumin in the experiments *in vitro* and *in vivo* showed that the isolated microsomes suffered a relatively greater loss in their ability to synthesize albumin than insoluble protein.

6. It is concluded that the results are consistent with the hypothesis that isolated rat-liver microsomes are able to synthesize albumin but that this albumin is not released in a soluble form.

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Metabolism of Polycyclic Compounds

15. THE CONVERSION OF NAPHTHALENE INTO A DERIVATIVE OF GLUTATHIONE BY RAT-LIVER SLICES*

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Naphthalene is converted by animals into a compound which is believed to be *N*-acetyl-*S*-(1:2-dihydro-2-hydroxy-1-naphthyl)-*L*-cysteine (scheme I, III), which gives rise to 1-naphthylmercapturic acid (VI) on acidification (Boyland & Sims, 1958). Knight & Young (1958) found that benzene, an-

thracene and the monohalogenobenzenes were probably converted into analogous derivatives. The mode of formation of these acids in the body has not yet been established, but they may arise by the reaction of intermediates (such as epoxides) with an SH-containing compound. In the formation of mercapturic acids, cysteine, glutathione and the cysteine SH of tissue protein have all been cited

* Part 14: Sims (1959).