- Riggs, A. F. (1953). *J. gen. Physiol.* **36**, 1.
Rossi-Fanelli, A., Antonini, E. & Caputo, A. (1958).
Biochim. biophys. Acta, **30**, 608.
Rossi-Fanelli, A., Antonini, E. & Caputo, A. (1961a).
J. biol. Chem. **236**, 391.
Rossi-Fanelli, A., Antonini, E. & Caputo, A. (1961b).
J. biol. Chem. **236**, 397.
Schachman, H. K. (1960). *Brookhaven Symp. in Biology*,
no. 13, p. 49. New York: Brookhaven National Laboratory.
Svedberg, T. & Pedersen, K. O. (1940). *The Ultracentrifuge*.
Oxford: Clarendon Press.
Theorell, H. (1938). *Biochem. Z.* **298**, 242.
Yphantis, D. A. (1960). *Ann. N.Y. Acad. Sci.* **88**, 586.

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The Incorporation of [^{14}C]Leucine into Serum Albumin by the Isolated Microsome Fraction from Rat Liver

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It has previously been shown that, when the isolated microsome fraction from rat liver is incubated under suitable conditions with [^{14}C]amino acid, the serum albumin which is subsequently released from the particles by ultrasonic vibrations is radioactive (Campbell, Greengard & Kernot, 1960). This finding is in accord with the synthesis of serum albumin by the isolated microsome preparation. However, before this hypothesis can be advanced with certainty, more information is required about the way in which the [^{14}C]amino acid becomes bound in peptide linkage.

In the previous experiments the serum albumin released from the particulate fraction of the incubation mixture was diluted with carrier-inactive albumin. It was then purified by various methods including electrophoresis on cellulose acetate. The experiments have now been repeated on a larger scale with [^{14}C]leucine of a high specific radioactivity. Sufficient albumin was obtained to obviate the necessity for the use of carrier. The purified ^{14}C -labelled serum albumin was then subjected to hydrolysis with trypsin and the peptides were separated by high-voltage ionophoresis. A radioautograph revealed the pattern of radioactive peptides obtained. This pattern has been compared with that obtained from a sample of albumin labelled with [^{14}C]leucine *in vivo*. The two patterns, although differing in some respects, show marked similarities. Whereas the results obtained are in accord with the synthesis of serum albumin in the isolated microsome system, a complete interpretation must await a fuller understanding of the way in which the polypeptide chain of albumin is built up.

Some aspects of this work have been previously reported (Campbell, 1961; Campbell, Kernot & Smith, 1961).

MATERIALS AND METHODS

Abbreviation. Phosphoenolpyruvate, PEP.

Chemicals. The dipotassium salt of ATP was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Pyruvate kinase and the silver barium salt of PEP were obtained from C. F. Boehringer und Soehne, Mannheim, Germany. Solutions of the free acid were prepared before use and adjusted to pH 7.5 (indicator paper) with KOH. The disodium salt of PEP was kindly donated by Dr V. M. Clarke. Tris was obtained from British Drug Houses Ltd., Poole. Crystalline trypsin was obtained from Worthington Biochemical Corp., N.J., U.S.A.

Radioactive amino acids. Uniformly labelled L-[^{14}C]leucine with a specific radioactivity of 6 mc/m-mole was obtained from The Radiochemical Centre, Amersham, Bucks., and with a specific radioactivity of 90 mc/m-mole from Institut Pasteur, Paris, France.

The purity of the Institut Pasteur [^{14}C]leucine was checked by mixing a small quantity (0.5 μC) with a mixture of many amino acids and submitting it to two-dimensional paper partition chromatography, with phenol-NH₃-water and butanol-acetic acid-water (4:1:5) as solvents. A radioautograph revealed the presence of three radioactive substances other than leucine. These did not appear to be amino acids. The presence of isoleucine in the [^{14}C]leucine was checked by paper chromatography with water-saturated *tert.*-amyl alcohol (2-methylbutan-2-ol) as solvent (Work, 1949). No radioactive isoleucine was detected.

The above-mentioned experiments indicated that all the radioactive impurities could be removed from the [^{14}C]leucine by one-dimensional chromatography in the butanol-acetic acid-water solvent. Since it was judged that the contamination of the [^{14}C]leucine by other substances might account for as much as 10% of the radioactivity, the [^{14}C]leucine was purified as follows. Whatman no. 3 paper was pretreated by washing first with N-Na₂CO₃, then with water, 2N-acetic acid and finally water. Ordinary descending chromatographic procedures were used for this washing, each solvent being run for 24 hr. The [^{14}C]leucine (180 μC) was then applied to the paper, which was developed with the butanol-acetic acid-water solvent over-

night. After drying the paper, the position of the leucine was determined by radioautography, the paper was cut and the leucine eluted with water. This [^{14}C]leucine was used in all the metabolic experiments. At the completion of the experiments a sample of the purified [^{14}C]leucine was subjected to two dimensional paper chromatography and shown to be uncontaminated by other radioactive substances.

Animals. Rats were Wistar-strain albinos highly inbred at this Institute. Partial hepatectomy was performed by the method of Higgins & Anderson (1931). Rats with a weight of about 150 g. were killed by a blow on the head before decapitation.

Preparation of rat serum albumin and antiserum. These were prepared as described by Campbell *et al.* (1960).

Injection of [^{14}C]leucine in the whole animal. The amount of [^{14}C]leucine required was dissolved in 0.5 ml. of 0.65 M-phosphate buffer, pH 7.8. The solution was then injected intravenously through the vein of the penis of a rat (200–210 g. body wt.) under light ether anaesthesia.

Preparation of microsomes. All preparations were carried out between 0° and 4°. Microsomes were prepared by a method which followed closely that of Zamecnik & Keller (1954). The liver was homogenized in 2.5 times its weight of a medium containing 0.01 M-MgCl₂, 0.025 M-KCl, 0.035 M-tris buffer, pH 7.8, and 0.15 M-sucrose (Rendi & Hultin, 1960). The tissue suspension was centrifuged for 10 min. at 12 000g to remove cell debris, nuclei and mitochondria. The supernatant was centrifuged in a Spinco model L preparative centrifuge (no. 40 rotor) for 50 min. at 105 000g. In the experiments in which the proteins were labelled *in vivo* the supernatant was discarded after this centrifuging and the microsome pellet was treated with ultrasonic vibrations (see below). In the experiments in which the isolated microsomes were to be incubated with [^{14}C]leucine some of the clear supernatant was siphoned off with a syringe to provide the cell-sap fraction, 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.

Method of incubation. The incubations were carried out in four 100 ml. conical flasks, each of which contained 4 ml. of the microsome suspension, 2 ml. of cell sap, 20 μmoles of ATP, labelled amino acid, 100 μmoles of PEP and 500 $\mu\text{g.}$ of pyruvate kinase, in a total volume of 10 ml. The contents were gently shaken in air at 37° for 50 min. After incubation the microsomes were isolated from the incubation mixture by further centrifuging at 105 000g 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. The microsomes from 8 g. of liver were suspended in 10 ml. of 0.9% NaCl by gentle homogenization. The suspension was subjected to ultrasonic vibrations for 2 min. by a slight modification of the method previously described (Campbell *et al.* 1960).

A titanium probe (narrow end 1.9 cm. diam., 6 cm. long; wide end 3 cm. diam., 6 cm. long) was used. The

particulate suspension was placed in a glass tube (2.7 cm. diam.) which was held in an ice bath. The titanium probe was placed just below the surface of the suspension. After subjection to ultrasonic vibrations the suspension was stored at -15°, thawed and centrifuged at 90 000g for 60 min. to remove insoluble material.

The soluble extract was freeze-dried and then extracted with phosphate buffer and ethanol as described by Peters (1958). To the dry extract (from up to 40 g. of liver) 1 ml. of 0.05 M-sodium phosphate buffer, pH 2.4, was added. To the suspension was then added 4 ml. of 80% ethanol, the pH of which had been adjusted to 2.4 by the addition of N-HCl. The mixture was gently shaken and after 15 min. centrifuged to give a clear supernatant, which was dialysed against distilled water overnight. The precipitate that formed on dialysis was removed by centrifuging and the clear supernatant (about 9 ml.) was freeze-dried from a 25 ml. round-bottom flask with a R24 standard ground-glass joint as described previously (Campbell *et al.* 1960).

Zone electrophoresis of the freeze-dried protein on cellulose acetate was then performed as previously described (Campbell *et al.* 1960). After extraction of the albumin from the cellulose acetate with 0.9% NaCl, a small sample was removed for the determination of radioactivity and the remainder of the albumin was denatured by the addition of sufficient trichloroacetic acid to give a final concentration of 5% (w/v), and by heating at 90° for 20 min. The fine precipitate of albumin was centrifuged down and washed with acetone once and ether twice.

Hydrolysis and electrophoresis of purified albumin. The albumin (200–250 $\mu\text{g.}$) was oxidized with performic acid under the conditions of Hirs (1956). It was then hydrolysed with trypsin. An amount of trypsin equal to 1% of the weight of protein was added in 0.2 ml. of 1% ammonium carbonate and the whole incubated for 12 hr. at 37°, and freeze-dried.

The albumin hydrolysate was dissolved in a small volume of water and applied to a strip of Whatman no. 1 paper. Ionophoresis was performed in a pyridine-acetate buffer, pH 3.5, as described by Ryle, Sanger, Smith & Kitai (1955), at 30 v/cm. for 2 hr.

After ionophoresis the paper was dried and a radioautograph prepared with X-ray film (Ilford Industrial G).

Measurement of radioactivity. (1) Trichloroacetic acid-precipitated proteins. The extracted proteins were placed on 0.28 cm.² perspex disks and counted at infinite thickness with a thin mica end-window (2 mg./cm.²) Geiger-Müller counter. A standard planchet of 1 cm.², containing 1 μC of $^{14}\text{C/g.}$, gave approx. 1000 counts/min. under these conditions. (2) Albumin. The radioactivity was determined as described by Campbell & Stone (1957).

RESULTS

Methods for the extraction of serum albumin from the microsome fraction

In previous experiments (Campbell *et al.* 1960) in which albumin was isolated from the microsome extracts after the addition of carrier inactive albumin the trichloroacetic acid-ethanol procedure of Debro, Tarver & Korner (1957) was used. In the present experiments it was necessary to obtain as complete a recovery as possible of the serum

albumin in the extracts since it was undesirable to dilute the radioactive albumin by the addition of carrier. Whereas the trichloroacetic acid method provides albumin of a high purity the yield from the microsomal extracts was very low. In practice, unless 1 mg. of albumin was obtained from the ethanol extract there was insufficient albumin after electrophoresis for the subsequent hydrolysis with trypsin.

Very much higher yields of albumin were obtained by the use of phosphate buffer, pH 2.4, and ethanol (Peters, 1958). With this method the yield of albumin in the ethanolic solution was nearly quantitative. Electrophoresis showed that the albumin obtained after freeze-drying was usually contaminated with a small quantity of two other proteins with the mobility of α_1 - and β_1 -globulin respectively, but these were completely eliminated by the use of zone electrophoresis on cellulose acetate.

An attempt was made to eliminate the ultrasonic stage of the method and to extract albumin from the microsomal pellet by the direct addition of phosphate and ethanol. The yield of albumin by this procedure was, however, considerably less than when the ultrasonic extract was treated with phosphate and ethanol.

The yield of albumin from the microsome extract from 8 g. (wet wt.) of rat liver was 1.0–1.2 mg. in the dialysis residue after ethanol extraction. After freeze-drying and electrophoresis the yield was 250–350 μ g. This was an ideal amount of protein for treatment with trypsin and high-voltage ionophoresis of the peptides.

Optimum conditions for incorporation of [14 C]leucine into microsomal protein from regenerating liver

In order to obtain albumin of the required specific radioactivity it was necessary to determine the optimum conditions for the incorporation of [14 C]leucine into the microsomal protein. PEP was a better energy source than 3-phosphoglycerate. The addition of pyruvate kinase had a slight stimulatory effect and so was included in the medium.

Another important factor in obtaining highly radioactive serum albumin was to ensure that the [14 C]leucine in the incubation medium had a high specific radioactivity. Preliminary experiments indicated that when the microsomes from regenerating liver were incubated under the conditions given in the Materials and Methods section the incubation mixture contained about 5 μ g. of free leucine/ml. For this reason the amount of cell sap present in the incubation mixture was kept to a minimum. In the first experiments in which [14 C]leucine with a specific radioactivity of 6 mc/m-mole was used the radioactivity of the microsomal protein after incubation showed only a small increase as progressively increasing amounts

of [14 C]leucine were included in the incubation mixture. When [14 C]leucine with a specific radioactivity of 90 mc/m-mole was substituted, the effect of increasing quantities in the incubation mixture was more marked. When the mixture contained 4 μ C of [14 C]leucine/ml. there was a ninefold increase in the radioactivity of the microsomal proteins when the amino acid with the higher specific radioactivity was used.

Radioactivity of microsomal albumin obtained in vivo and in vitro

Two series of experiments were performed, first with [14 C]leucine of low specific radioactivity and secondly with that of higher activity. The specific radioactivity of the serum albumin released by ultrasonics was determined, after purification by electrophoresis on cellulose acetate, by the addition of antiserum as previously described (Campbell *et al.* 1960). When 50 μ C of [14 C]leucine was injected and the rat killed after 30 min., the radioactivity of the microsomal albumin was 6770 counts/min./mg. of albumin when the specific radioactivity of the [14 C]leucine was 6 mc/m-mole, and 6751 counts/min. when the injected [14 C]leucine had an activity of 90 mc/m-mole. As expected, the amino acid with higher specific activity did not influence the radioactivity of the albumin obtained from the liver microsomes under conditions *in vivo*.

When albumin was isolated from regenerating liver microsomes after their incubation with 5 μ C of the low-activity [14 C]leucine/ml. of incubation mixture the specific radioactivity of the albumin was 1767 counts/min./mg. When 3 μ C of the high-activity [14 C]leucine/ml. was used the corresponding activity of the albumin was 11 200. Hence the higher specific activity amino acid had the expected beneficial effect on the activity of the isolated albumin.

Pattern of incorporation of [14 C]leucine into serum albumin

Although the initial experiments with the low specific activity [14 C]leucine provided radioautographs of the trypsin hydrolysates of [14 C]albumin which indicated that many radioactive peptides were present, attention will be confined to the results obtained with the higher activity albumin. Only in this case were the hydrolysates from the experiments *in vivo* and *in vitro* run together on the same ionophoretic strip of paper. The results are shown in Plate 1. This shows that with both samples of albumin, over 20 bands of radioactivity, each probably representing several peptides containing [14 C]leucine, were present. Except in one or two cases the bands join up, indicating that the same peptide bands contained [14 C]leucine when the albumin was labelled either *in vivo* or *in vitro*.

Radioautograph obtained after ionophoresis (pH 3.5; 30 v/cm.; 2 hr.) of trypsin hydrolysates of [^{14}C]leucine-labelled serum albumin. (a) Albumin labelled *in vitro*; (b) albumin labelled *in vivo*.

There are, however, marked differences between the extent of labelling of the peptide bands obtained under the two conditions.

DISCUSSION

A protein, such as serum albumin, could acquire radioactivity from [^{14}C]amino acids in a system *in vitro* in several ways. First, there might be a linkage between [^{14}C]amino acid and the ϵ -amino groups of lysine or the γ -carboxyl groups of glutamic acid or aspartic acid. Secondly, some of the amino acid residues in the polypeptide chain might exchange with the free [^{14}C]amino acid without there being a net increase in the number of polypeptide bonds. Thirdly, incorporation may be due to the formation of new polypeptide chains. This may occur either by the completion of partially complete chains or by the synthesis of completely new ones. Dintzis (1961) has produced evidence that in the intact reticulocyte haemoglobin is synthesized by the stepwise extension of polypeptide chains, and Bishop, Leahy & Schweet (1960) have shown that with isolated ribosomes most of the incorporation of [^{14}C]valine into haemoglobin is due to the completion of partial chains.

In the present experiments it is assumed that most of the incorporation of [^{14}C]leucine into serum albumin under conditions *in vivo* is due to protein synthesis. In order to increase the probability of this being so the period between injection and removal of the liver was prolonged to 30 min. On this basis a comparison between the peptides labelled under conditions *in vivo* and *in vitro* should reveal the extent to which protein synthesis is taking place during the incubation of the isolated microsome fraction.

Examination of Plate 1 shows that under both conditions of labelling approximately 21 peptide bands derived from the tryptic hydrolysis of albumin contained [^{14}C]leucine. From the known content of arginine and histidine in the serum albumin of several species more than 21 peptides would be expected to result from tryptic hydrolysis. The discrepancy is no doubt due to the comparatively poor resolution of the single-dimensional electrophoresis, so that each band could contain up to two to three [^{14}C]leucine peptides. Owing to the low amount of radioactivity available it was not possible to detect the presence of radioactive peptides after a two-dimensional fractionation, so that the best possible single-dimensional system was chosen.

A comparison between the bands labelled under the two conditions shows that in virtually every case the same bands were labelled. In spite of this there are marked differences between the relative extent to which the various peptide bands were labelled. There are a number of possible reasons for

this, some of which can be discounted. First the differences might be due to differences in the extent of hydrolysis of the two samples. This seems unlikely since very similar patterns were obtained when different samples of rat serum albumin were hydrolysed independently. In the present experiments great care was taken to ensure that both samples were hydrolysed under identical conditions so that even if hydrolysis were not complete similar patterns would have been expected. Secondly it might be suggested that the patterns obtained were not due to serum albumin itself but to some radioactive contaminant. This seems unlikely, for care was taken to purify the albumin by methods which have been found satisfactory in previous experiments (Campbell *et al.* 1960). More important is that quite different patterns are obtained from the hydrolysis of different proteins and even of serum albumins from different species of animal. In one set of experiments microsomal albumin was labelled with [^{35}S]amino acids *in vivo*. Two such samples of albumin were obtained, one with a high specific radioactivity and one with a low. When the high-activity albumin was diluted with carrier serum albumin to bring the two samples to the same specific radioactivity the peptide patterns obtained after hydrolysis were identical. Such a result would not have been obtained if the labelling patterns were due to contamination.

Owing to the comparatively low resolution of the single-dimension electrophoresis, it remains a possibility that the differences between the patterns *in vivo* and *in vitro* are due to differences in the labelling of the various peptides contained within each band. This cannot be disproved until better resolution is obtained, but it seems an unlikely explanation since the bands in question were generally labelled more heavily under conditions *in vitro* than *in vivo*. A further possible explanation is that the differences are due to the changes in the radioactivity of the pool of free [^{14}C]leucine, which will be greater under the conditions *in vivo* than *in vitro*. Further experiments are required to evaluate this hypothesis.

While therefore the present results are consistent with the hypothesis that the isolated microsome fraction of rat liver is able to synthesize serum albumin, it is not clear at present to what extent completely new polypeptide chains are synthesized under these conditions.

SUMMARY

1. A method is described for the extraction of serum albumin from microsomal extracts in high yield.
2. The microsomal fraction from regenerating rat liver was incubated with [^{14}C]leucine and a

source of high-energy phosphate. The particles were separated by centrifuging and serum albumin was released by ultrasonic vibrations. The albumin was purified finally by zone electrophoresis on cellulose acetate.

3. A rat was intravenously injected with [^{14}C]-leucine and after 30 min. the animal was killed. Albumin was isolated from the microsome fraction of the liver.

4. The radioactive albumin samples obtained under conditions *in vitro* and *in vivo* were denatured, oxidized and hydrolysed with trypsin. The peptides resulting were fractionated by ionophoresis on paper and the [^{14}C]leucine-containing peptides located by radioautography.

5. Comparison of the radioautographs indicated about 20 bands of active peptides in each case. The peptides labelled were in general similar under the two conditions but the degree of labelling showed marked differences.

6. The results are consistent with the synthesis of serum albumin by the isolated microsome fraction. They appear to exclude the incorporation of [^{14}C]leucine into serum albumin by attachment to the residues of the basic or acidic amino acids or by a limited exchange with leucine residues. The extent to which entirely new polypeptide chains are synthesized under the conditions *in vitro* is not at present known.

It is a pleasure to acknowledge the assistance of Dr F. Sanger, F.R.S., and Dr L. F. Smith in the aspects of the work involving the trypsin hydrolysis of the albumin

samples and the ionophoresis of the peptides. We are also grateful for their interest and valuable criticisms. We should also like to thank Professor F. Dickens, F.R.S., for his interest, Dr J. K. Whitehead for assistance with the purification of the [^{14}C]leucine and Mr H. E. H. Jones for performing the partial hepatectomies and the intravenous injections. This work was made possible by a grant to the Medical School from the British Empire Cancer Campaign.

REFERENCES

- Bishop, J., Leahy, J. & Schweet, R. (1960). *Proc. nat. Acad. Sci., Wash.*, **46**, 1030.
 Campbell, P. N. (1961). In *Protein Biosynthesis*, p. 27. Ed. by R. J. C. Harris. London: Academic Press.
 Campbell, P. N., Greengard, O. & Kernot, B. A. (1960). *Biochem. J.* **74**, 107.
 Campbell, P. N., Kernot, B. A. & Smith, L. F. (1961). *Biochem. J.* **81**, 25P.
 Campbell, P. N. & Stone, N. E. (1957). *Biochem. J.* **66**, 19.
 Debro, J. R., Tarver, H. & Korner, A. (1957). *J. Lab. clin. Med.* **50**, 728.
 Dintzis, H. M. (1961). *Proc. nat. Acad. Sci., Wash.*, **47**, 247.
 Higgins, G. M. & Anderson, R. M. (1931). *Arch. Path.* **12**, 186.
 Hirs, C. H. W. (1956). *J. biol. Chem.* **219**, 611.
 Peters, T., jun. (1958). *J. Amer. chem. Soc.* **80**, 2700.
 Rendi, R. & Hultin, T. (1960). *Exp. Cell Res.* **19**, 253.
 Ryle, A. P., Sanger, F., Smith, L. F. & Kitai, R. (1955). *Biochem. J.* **60**, 541.
 Work, E. (1949). *Biochim. biophys. Acta*, **3**, 400.
 Zamecnik, P. C. & Keller, E. B. (1954). *J. biol. Chem.* **209**, 337.
 Zamecnik, P. C., Loftfield, R. B., Stephenson, M. L. & Steele, J. M. (1951). *Cancer Res.* **11**, 592.

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Sulphydryl Groups of the Uterus of the Ovariectomized Rat after Treatment with Oestradiol

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Scott & Lisi (1960) reported the changes in activities of some enzymes of extracts of the uteri of ovariectomized rats after injection of oestradiol-17 β . In order to be sure that the changes noted could not have been affected by the oxidation of TPNH $_2$ by oxidized glutathione in the extracts, a few extracts were titrated for acid-soluble sulphydryl-group content. The concentration of sulphur reducible to sulphydryl groups was found not to be sufficient to affect the enzyme determinations. However, to be sure this was a valid deduction at all times considered in the enzyme study, another series of animals were investigated

to determine any changes in the sulphydryl-group concentration and content of the uterus after injection of oestradiol. Changes in sulphydryl groups which were parallel to the changes in enzyme activities are reported.

MATERIALS AND METHODS

Chemicals. TPN, monosodium salt, and glucose 6-phosphate, disodium salt, were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. GSH and GSSG were obtained from Schwarz Laboratories Inc., Mount Vernon, N.Y., U.S.A. All water was doubly distilled, the second time in an all-glass still.