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The Sequential Synthesis of the Polypeptide Chain of Serum Albumin by the Microsome Fraction of Rat Liver

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1. The isolated microsome fraction of regenerating rat liver was incubated with cell sap, a source of energy and [^{35}S]methionine, [^{14}C]isoleucine or [^{14}C]leucine for different periods of time, and microsomal albumin isolated. 2. The distribution of these isotopes in albumin was determined by separation of tryptic peptides from the protein. Radioactivity was measured in peptides either qualitatively by radioautography or quantitatively by labelling with both ^{3}H and ^{14}C. 3. A gradient of radioactivity existed at all times in albumin isolated after incubating microsomes. 4. The shorter the incubation time the fewer the peptides labelled in albumin, but the peptides with highest specific activity after short incubation times corresponded to those with highest specific activities after long incubation times. 5. Leucine released from the *C*-terminus of albumin had a higher specific activity than the mean specific activity of the remaining leucine residues in albumin. 6. The peptide with the highest specific activity in albumin is probably derived from the *C*-terminus of the protein. 7. [^{14}C]Glutamic acid is incorporated into the *N*-terminus of albumin after incubating the microsome fraction with this isotopically labelled amino acid, cell sap and a source of energy. The specific activity of the *N*-terminal glutamic acid under these conditions is less than the mean specific activity of the remaining glutamic acid and glutamine residues in albumin. 8. The results are interpreted as reflecting a sequential synthesis of serum albumin in the isolated microsome fraction of rat liver. The direction of synthesis of albumin is from the *N*-terminus towards the *C*-terminus. 9. The bulk of incorporation of radioactive amino acid into albumin in the isolated microsome fraction is due to completion of partially completed, pre-existing peptide and polypeptide chains. A limited synthesis of new chains of albumin does, however, occur.

Dalgliesh (1953) first proposed that the synthesis of a protein occurred by a mechanism whereby several polypeptide chains representing different stages in the completion of the complete protein were attached simultaneously to a nucleic acid template. These chains were considered to grow by the stepwise addition of amino acids so that growth of the protein proceeded in a linear manner from one terminus to the other. This hypothesis predicts that the subcellular fraction where protein synthesis takes place, i.e. the microsome fraction or ribosomes, will contain at any given time a spectrum of polypeptide chains in differing degrees of completion (Fig. 1). If the synthesis takes place in the presence of a radioactive amino acid with a reasonably constant specific radioactivity it is predicted that, after short periods of synthesis (*A* in Fig. 1), most of the radioactivity will be confined to that part of the chain which is finished last. More extensive synthesis will lead to progressively more of the isotope being located in that part of the chain where growth starts (*B* in Fig. 1). Provided, therefore, that a large number of complete chains are not synthesized from end to end a substantial gradient of activity will always exist from the terminus where growth starts towards the terminus where growth finishes.

That a protein can be synthesized by the above mechanism was shown first by Bishop, Leahy & Schweet (1960) in a study of the biosynthesis of haemoglobin by ribosomes isolated from rabbit reticulocytes. They showed further that the direction of synthesis of haemoglobin was from the *N*-terminus towards the *C*-terminus, this conclusion being fully substantiated in subsequent work by Dintzis (1961) and by Naughton & Dintzis (1962) with intact rabbit reticulocytes. Essentially similar results have been obtained for the biosynthesis of a

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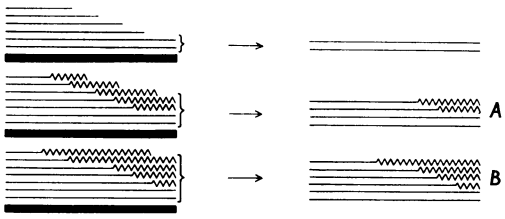

Fig. 1. Predicted distribution of radioactivity in an amino acid-incorporating system according to a mechanism of sequential synthesis of protein. The isolated microsome fraction contains a spectrum of non-radioactive peptide and polypeptide chains (—) in different stages of completion attached to a template (■). Amino acid incorporation takes place in the presence of cell sap, a source of energy and a radioactive amino acid such that radioactivity is incorporated into the chains (∿). As indicated by the brace (}) only completed chains will be isolated as the intact protein. *A* represents incorporation for a relatively short time, and *B* represents incorporation for a relatively long time.

bacterial amylase (Yoshida & Tobita, 1960), for the synthesis of lysozyme in minced hen oviduct (Canfield & Anfinsen, 1963), for the synthesis of ribonuclease in pancreas slices (Luck & Barry, 1964) and for the synthesis of total protein in *Escherichia coli* (Goldstein & Brown, 1961). Jungblut (1963) perfused an isolated liver of a rat and produced evidence that the polypeptide chain of serum albumin was completed at the *C*-terminus.

Previous work supports the idea that the isolated microsome fraction of rat liver can synthesize serum albumin (Campbell, Greengard & Kernot, 1960; Campbell & Kernot, 1962). The present studies are an extension of the latter work and furnish evidence that the isolated microsome fraction of rat liver can synthesize the complete chain of serum albumin, albeit to a limited extent. Most of the incorporation of amino acid in the microsomal system appears to be due to the completion of pre-existing, partially completed peptide chains. The mechanism of synthesis is sequential and the direction of growth is from the *N*-terminus towards the *C*-terminus. Brief accounts of these results have already been presented (Sargent & Campbell, 1963, 1964).

MATERIALS AND METHODS

Radioactive chemicals. L-[U-^{14}C]Leucine (specific activity 45–47 or 198mc/m-mole), L-[U-^{14}C]glutamic acid (specific activity 39mc/m-mole) and DL-[4,5-$^{3}H_2$]leucine (specific activity 5000 or 10900mc/m-mole) were obtained from The Radiochemical Centre, Amersham, Bucks. L-[U-^{14}C]Leucine (specific activity 198mc/m-mole) and L-[U-^{14}C]isoleucine (specific activity 246mc/m-mole) were purchased from the New England Nuclear Corp., Boston, Mass., U.S.A.

[^{35}S]Methionine was isolated from yeast grown on $^{35}SO_4^{2-}$ by a method based on that of Williams & Dawson (1952). The medium used was exactly as described by these authors except that the $MgSO_4,7H_2O$ was replaced by $MgCl_2,6H_2O$ (final concn. 20·5mg./l.). Before use this medium was diluted with an equal volume of a solution of Na_2SO_4 (83mg./l.). The diluted growth medium (150ml.) containing 20mc of $^{35}SO_4^{2-}$ was inoculated with 5ml. of a suspension of commercial baker's yeast (1·0g. wet wt.) in 0·9% NaCl and growth was allowed to proceed at 30° for 2 days with vigorous shaking. After this time the yeast was collected by centrifuging and was washed exhaustively with 0·9% NaCl. Yeast protein was isolated after precipitation with trichloroacetic acid according to a modified Schneider procedure (Zamecnik, Loftfield, Stephenson & Steele, 1951). The dried yeast protein was hydrolysed by heating under reflux with constant-boiling HCl (twice glass-distilled) for 24hr. and [^{35}S]methionine isolated from the hydrolysate by chromatography on Zeo-Karb 225 (H^+ form) with a gradient of 1–2N-HCl as eluent. The fraction containing [^{35}S]methionine was evaporated to dryness under vacuum and the final purification was by chromatography on Whatman no. 3 paper with butan-1-ol–acetic acid–water (50:11:25, by vol.) as solvent. The approximate specific activity of the purified [^{35}S]methionine was 430mc/m-mole. This product was stored in the solid state at room temperature under vacuum for up to 2 months. Routine checks by paper chromatography failed to reveal significant amounts of radiochemical decomposition products.

Other chemicals. Phosphoenolpyruvate was synthesized as the cyclohexylammonium salt by the method of Clark & Kirby (1963). Cyclohexylamine was found to be inhibitory to amino acid incorporation in the isolated rat-liver microsome system and the compound was therefore converted into the silver–barium salt, before storage, in the following way. Cyclohexylammonium phosphoenolypyruvate (2g.) was dissolved in 30ml. of 0·2N-HNO_3. Then 30ml. of a solution of 2·1g. of $Ba(NO_3)_2$ in water was added followed by 5ml. of a solution of 1·3g. of $AgNO_3$ in water. The pH of the solution was adjusted to 7·4 with 5N-NH_3 and the precipitate collected by centrifuging. After washing with water, aq. 50% (v/v) ethanol, ethanol and ether the salt was dried over P_2O_5 under vacuum in the dark. The dipotassium salt of ATP was purchased from the Sigma Chemical Co., St Louis, Mo., U.S.A. Solutions of phosphoenolpyruvate and ATP were prepared as their potassium salts as described by Campbell *et al.* (1960). Tris (specially purified by the manufacturers) was purchased from British Drug Houses Ltd., Poole, Dorset. The pH of all solutions containing tris was adjusted at room temperature (about 20°). All other inorganic components of incorporation media were AnalaR chemicals and all solutions were prepared in twice-glass-distilled water.

2,5-Diphenyloxazole, 1,4-bis-(5-phenyloxazol-2-yl)benzene and naphthalene, all of scintillation grade, were purchased from Nash and Thompson Ltd., Tolworth, Surbiton, Surrey. Aerosil standard silica for use in scintillation counting was purchased from Busch, Beach and Segner Bayley Ltd., London, E.C. 3. Toluene, dioxan and ethanol used in scintillation media were all of AnalaR standard.

Trypsin, a three-times-crystallized salt-free sample (lot no. 763), and carboxypeptidase A–DFP (lot no. 6127) were obtained from Worthington Biochemical Corp., Freehold,

N.J., U.S.A. The latter was a preparation of twice-crystallized carboxypeptidase A that had been treated with di-isopropyl phosphorofluoridate to eliminate residual tryptic and chymotryptic activity and finally crystallized after the removal of the excess of di-isopropyl phosphorofluoridate. Pyruvate kinase was obtained from Boehringer und Soehne G.m.b.H., Mannheim, Germany.

Animals. The rats used were Wistar-strain albinos from a closed colony bred at this Institute. Regenerating liver was obtained from rats 2 days after partial hepatectomy by the method of Higgins & Anderson (1931). Rats were killed by a blow on the head and decapitated, after which the livers were rapidly removed and chilled before isolation of the microsomes.

Preparation of the microsome fraction. The microsome fraction was isolated in a medium containing $MgCl_2$ (10mM), KCl (25mM), tris buffer (pH 7·8 at 20°) (35mM) and sucrose (0·15M) (Rendi & Hultin, 1960), as described by Campbell & Kernot (1962).

Incubation of the microsome fraction. The microsome fraction from 40g. of regenerating rat liver was incubated in air with gentle shaking in four separate 100ml. conical flasks at 37°, each flask containing 4ml. of microsome suspension, 2ml. of cell sap, 200μmoles of phosphoenolpyruvate, 20μmoles of ATP, 500μg. of pyruvate kinase and labelled amino acid, as detailed in individual experiments, in a total volume of 10ml. The reaction was stopped at the times stated in individual experiments by rapidly transferring the flasks to ice–water and swirling the contents for several minutes.

Isolation of albumin from the microsome fraction. After incubation the microsome fraction was isolated from the medium by centrifuging at 105000*g* for 60min.; albumin was isolated from the pellet by a method involving treatment with ultrasonic vibration, freezing and thawing, extraction with phosphate-buffered ethanol, pH 2·5, and electrophoresis on cellulose acetate in barbital buffer, pH 8·6, as described by Campbell & Kernot (1962).

After electrophoresis the albumin zone was eluted into 0·9% NaCl and a small sample removed to determine its specific radioactivity by precipitation with antibody (Campbell *et al.* 1960). The remaining albumin was denatured by heating at 90° for 20min. in trichloroacetic acid (5%, w/v) and the protein washed with acetone and ether before being air-dried. By this procedure 200–400μg. of microsomal albumin could be isolated from 40g. of regenerating liver.

Separation of the tryptic peptides from albumin. Before digestion with trypsin the albumin was oxidized with performic acid to rupture disulphide bonds by the method of Hirs (1956). Oxidized albumin (200μg.) was suspended in 0·2ml. of a solution of 1% (w/v) ammonium carbonate containing trypsin equivalent to 1% of the albumin by weight, and digestion allowed to proceed at 37° for 16hr. The oxidized protein became soluble within the first 15min. of digestion. The digest contained either a drop of toluene or a piece of filter paper saturated with toluene was suspended inside the tube to prevent contamination with bacteria and bacterial proteolysis. After incubation the solution was freeze-dried.

Tryptic peptides were dissolved in 1% (v/v) trimethylamine and applied to either Whatman no. 3MM or no. 1 papers of dimensions 57cm. × 6cm. Whatman no. 3MM paper was used with quantities of protein up to 2·5mg., and no. 1 paper was usually used when the quantities of protein did not exceed 1·0mg. Papers were washed with N-Na_2CO_3, water, N-acetic acid and finally water, and then air-dried before use. Electrophoresis was carried out in an apparatus of the type described by Ryle, Sanger, Smith & Kitai (1955), with Esso White Spirit 100 as coolant. A voltage of 40v/cm. was applied for 90min., with pyridine–acetic acid–water (1:10:89, by vol.), pH 3·5, as buffer. For two-dimensional separations the strip of paper containing the peptides was air-dried and then machine-sewed to a sheet of Whatman paper (57cm. × 34cm.). Papers were then subjected to ascending chromatography in butan-1-ol–acetic acid–water (3:1:1, by vol.) for 20hr. at room temperature.

Peptides were detected by dipping the papers in a solution of 0·4% (w/v) ninhydrin in acetone and allowing colour development to take place at room temperature overnight in the dark. In some cases radioactivity was detected on the papers by radioautography before development with ninhydrin. In this case the paper was sandwiched between two sheets of Kodak Blue Brand X-ray film. One of the X-ray films was developed after about 1 month and on the basis of the intensity of darkening of the film it was decided whether to leave the remaining film for a further 1, 2 or 3 months.

Elution of peptides from 'fingerprints'. In some experiments peptides were eluted from the paper before measurement of ^{14}C and ^{3}H activity. After detection of the peptides with ninhydrin the papers were washed with acetone and ether to remove excess of ninhydrin, and peptide areas cut from the paper and placed in 20ml. scintillation counting vials containing 5·0ml. of 5N-NH_3. The vials were held at 90° for 60min. after which the papers were removed with forceps. The residual solution in the vials was evaporated to dryness at 90°.

Digestion of albumin with carboxypeptidase A–DFP. Albumin (10mg.) was denatured by heating at 90° for 20min. in the presence of 5% (w/v) trichloroacetic acid. The dried protein was rapidly dissolved by adding 0·1ml. of 0·1N-NaOH followed by 0·8ml. of 1% (w/v) $NaHCO_3$ and 0·1ml. of 0·1N-HCl. To this solution was added 0·1ml. of a solution of carboxypeptidase A–DFP (1·0mg./ml.) in 1% (w/v) $NaHCO_3$ prepared by the method of Fraenkel-Conrat, Harris & Levy (1955). The solution was incubated at 37° for 16hr. and the reaction stopped by the addition of an equal volume of 10% (w/v) trichloroacetic acid. The precipitate was collected by centrifuging and washed with water, acetone and ether before being air-dried. The supernatant fraction from the digest was adsorbed on to a washed column of Zeo-Karb 225 (H^+ form) and the column washed thoroughly with water. Amino acids were eluted with N-NH_3 and the eluate was evaporated to dryness under vacuum over P_2O_5.

Isolation of the N-terminal and internal glutamic acid residues in albumin. Microsomal albumin eluted from cellulose acetate after electrophoresis was diluted by the addition of 10mg. of rat serum albumin isolated by the method of Debro, Tarver & Korner (1957). The albumin was heat-denatured, collected by centrifuging and dissolved in 1% (w/v) $NaHCO_3$ as described for the carboxypeptidase digestion. The solution was treated with 1-fluoro-2,4-dinitrobenzene and the DNP-albumin isolated by the method of Sanger (1949). The DNP-albumin was hydrolysed in a sealed evacuated tube with 1·0ml. of constant-boiling

twice-distilled HCl at 110° for 24hr. The liberated DNP-glutamic acid was extracted into ether, the ether phase washed exhaustively with water and the DNP-glutamic acid purified by electrophoresis on Whatman no. 1 paper with pyridine–acetic acid–water (5:2:493, by vol.), pH 5·3, as buffer. Electrophoresis was carried out at 40v/cm. on a high-voltage electrophoresis apparatus with cooled metal plates as described by Gross (1961). The DNP-glutamic acid zone was eluted with methanol and the solution evaporated to dryness.

The aqueous phase remaining after hydrolysis of the DNP-albumin was evaporated to dryness repeatedly and the free amino acids were converted into their DNP derivatives by reaction with 1-fluoro-2,4-dinitrobenzene. DNP-glutamic acid, representing all the glutamic acid and glutamine residues in albumin with the exception of the *N*-terminal glutamic acid residue, was isolated from the mixture by electrophoresis in pyridine–acetate buffer, pH 5·3, as described above. DNP-glutamic acid was estimated quantitatively from its extinction at 360mμ in N-NaOH by using a millimolar extinction coefficient of 17·4 (Fraenkel-Conrat *et al.* 1955).

Amino acid analysis. In one experiment an amino acid analysis was performed on rat serum albumin isolated by the method of Debro *et al.* (1957) and used without further purification. Albumin (4·2mg.) was hydrolysed with 1·0ml. of constant-boiling twice-distilled HCl in a sealed evacuated tube at 110° for 24hr. and the hydrolysates were repeatedly evaporated to dryness. Analysis of the mixture was performed on a Beckman–Spinco Automatic Amino Acid Analyser. We are grateful to Mrs Una Law of this Institute for this analysis.

Radioactivity measurements. (1) Solid-state counting. ^{14}C counting was performed as a routine with an automatic gas-flow-type counter (Nuclear–Chicago Corp. model C110B) with an efficiency of 22%. The methods for counting total microsomal proteins at infinite thickness and albumin at infinite thinness have been described by Campbell *et al.* (1960). In one experiment DNP-glutamic acid of low specific radioactivity was counted at infinite thinness on a low-background gas-flow type counter (Nuclear–Chicago Corp. model C115) with a background of 2 counts/min. DNP-glutamic acid samples were dissolved in methanol and applied to aluminium planchets of diameter 1in., and the methanol was removed under vacuum. A minimum of 2000 counts were collected to ensure accurate counting of the samples (Loftfield, 1963).

(2) Liquid-state counting. Protein and peptides with both ^{3}H and ^{14}C activity were counted in a liquid-scintillation spectrometer (Packard Tri-Carb model 314EX). The following counting procedure was used, the details of which were kindly supplied by Dr K. N. Jeejeebuoy, formerly of the Postgraduate Medical School, London. Dry samples in 20ml. scintillation vials were dissolved in 0·1ml. of 0·1N-NaOH and to each vial was added 20ml. of a solution containing 2,5-diphenyloxazole (7·0g.), 1,4-bis-(5-phenyloxazol-2-yl)benzene (150mg.), Aerosil standard silica (35g.), naphthalene (50g.), dioxan (800ml.), toluene (200ml.) and ethanol (30ml.). The vials were stoppered and shaken vigorously, when a gel formed within a few minutes. The vials were left at 2° overnight in the dark and bubbles that had formed in the gel after that time were removed by gently rotating the vial. Vials were then transferred to the counter and counted under the following conditions: high-voltage tap setting, 6/5/0; red channel 10–100 (gain 100%); green channel 10–100 (gain 10%). Under these conditions efficiencies, as determined by the internal standard method with [^{3}H]hexadecane and [^{14}C]hexadecane (purchased from The Radiochemical Centre, Amersham, Bucks.), were 18 and 21% for ^{3}H and ^{14}C respectively in the red channel and 3 and 70% for ^{3}H and ^{14}C respectively in the green channel. Backgrounds were 45 counts/min. in the red channel and 30 counts/min. in the green channel. The following equation was used to derive a ratio that is directly proportional to the ratio of the absolute number of disintegrations of ^{14}C and ^{3}H in the sample:

$$k\frac{^{14}C}{^{3}H} = \frac{x_1 - y_1 r}{y_2 r - x_2}$$

where x_1 represents the fraction of total ^{3}H counts in the red channel, y_1 the fraction of total ^{3}H counts in the green channel, y_2 the fraction of total ^{14}C counts in the green channel, x_2 the fraction of total ^{14}C counts in the red channel, r the ratio of the total counts in the red channel to total counts in the green channel, and k the constant of proportionality.

RESULTS

General considerations. The success of the present experiments depended critically on the ability to isolate from the radioactive proteins an amino acid containing sufficient radioactivity to allow the accurate determination of specific radioactivity. To achieve this objective attention was paid to two points, the source of the tissue extract and the specific radioactivity of the precursor amino acid.

We have previously shown that the microsome fraction from regenerating rat liver is more active for the incorporation of amino acid than is a similar fraction from normal liver. There is now evidence that such a fraction also has an enhanced activity for the synthesis of albumin. For this reason the microsome fraction from regenerating liver has been used throughout, although we believe the results obtained to apply also to normal liver (Campbell *et al.* 1960). Secondly, steps have been taken to ensure that the specific radioactivity of the free radioactive amino acid in the incubation medium was as high as possible. The amount of free leucine in 1ml. of our standard incubation mixture has been shown previously to be about 5μg. At this concentration of endogenous leucine a high specific radioactivity in the precursor leucine could be attained by adding large quantities of [^{14}C]leucine provided that the latter had a very high specific radioactivity. For this reason amino acids of high specific activity were used whenever possible. Even higher specific radioactivities in the incubation mixture could no doubt have been achieved by replacing the cell sap by a purified fraction. The aim of the work was to study the synthesis of albumin under optimum conditions. Since the complete requirements for the synthesis

of protein by the isolated microsome fraction are not known, the purification of the cell sap was not attempted as it was believed that the crude fraction was more likely to contain all the necessary factors.

Experiments designed to determine the effect of duration of synthesis on the pattern of amino acid incorporation. It was shown by Campbell & Kernot (1962) that albumin isolated after incubation of the microsome fraction with [^{14}C]leucine contains radioactivity in many of the tryptic peptides separated after high-voltage electrophoresis on paper. If the polypeptide chain were synthesized sequentially then the pattern of labelled peptides would be expected to change with respect to the duration of synthesis. To simplify the interpretation of the results it was decided to choose as the labelled amino acid one present in a relatively low amount in albumin. In this way the number of possible tryptic peptides containing the radioactive amino acid would be small. Table 1 shows the amino acid composition of rat serum albumin as determined by various authors. On the basis of these analyses it was decided to use [^{35}S]methionine and [^{14}C]isoleucine for the metabolic studies. Complete tryptic cleavage of the oxidized protein would yield at most six peptides containing methionine or 14 containing isoleucine.

Fig. 2 shows the incorporation of [^{14}C]isoleucine into total microsomal protein as a function of time. From these results it would be predicted that albumin would be maximally labelled at 30min. and it was decided to use 5 and 10min. as the intermediate times.

Isolation of microsomal albumin labelled with either [^{35}S]*methionine or* [^{14}C]*isoleucine.* The isolated microsome fraction from 40g. of regenerating rat liver was incubated with either [^{35}S]-methionine or [^{14}C]isoleucine as described in the Materials and Methods section and in Table 2. The times of incubation chosen were 30 and 8min. for [^{35}S]methionine and 30, 5 and 3·5min. for [^{14}C]isoleucine. Albumin samples were isolated from the microsomes after incubation as detailed in the Materials and Methods section and the

Table 1. *Amino acid composition of rat serum albumin*

Column (1) is from the results of Peters (1962*b*) and column (2) from the results of Jungblut & Turba (1963). Values in column (3) were determined as described in the Materials and Methods section and equated to those in columns (1) and (2) by assuming 6 methionine residues in albumin. —, Not determined.

Amino acid	Amino acid composition (residues/mol. of serum albumin) (1)	(2)	(3)
Asp	50	50	52
Thr	31	32	30
Ser	23	24	20
Glu	77	82	86
Pro	29	30	31
Gly	17	18	17
Ala	58	63	61
Cys (half)	30	37	32
Val	33	32	34
Met	6	6	6
Ile	13	14	14
Leu	53	55	59
Tyr	22	19	21
Phe	23	24	26
Lys	50	51	49
His	14	14	14
Arg	23	22	22
Trp	1	—	—
Amide N	38	41	—
Total	553	575	575

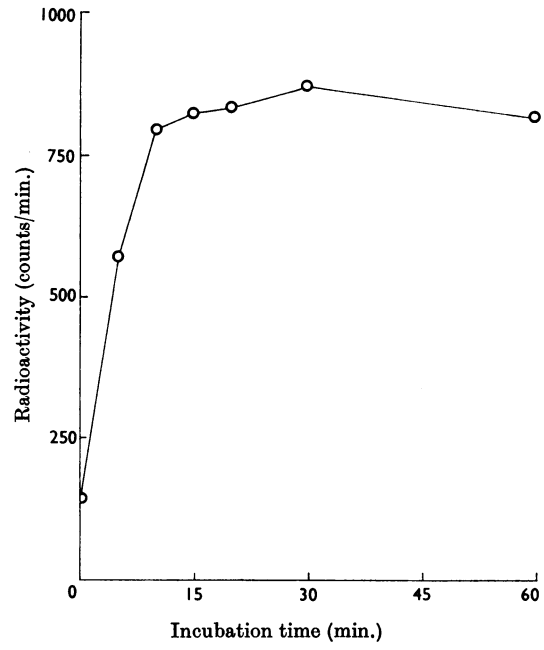

Fig. 2. Incorporation of [^{14}C]isoleucine into total microsomal protein as a function of time. A 0·4ml. sample of microsome suspension, 0·2ml. of cell sap, 20μmoles of phosphoenolpyruvate, 2μmoles of ATP, 50μg. of pyruvate kinase and 1·0μc of [^{14}C]isoleucine (specific activity 6mc/m-mole) were incubated in a total volume of 1·0ml. in the medium described in the Materials and Methods section. At the stated times the reaction was terminated by addition of 1·0ml. of 10% (w/v) trichloroacetic acid and total microsomal protein isolated. Radioactivity was determined at infinite thickness.

different yields and specific activities of the samples are shown in Table 2.

Distribution of [^{35}S]*methionine and* [^{14}C]*isoleucine in microsomal albumin.* Fig. 3 shows the distribution of radioactivity in albumin labelled with either [^{35}S]methionine or [^{14}C]isoleucine as determined by separation of tryptic peptides in a single dimension and detection of radioactivity by radioautography. Albumin isolated from the microsome fraction 30min. after incubation with [^{35}S]methionine shows about six radioactive zones with several other faint zones, whereas incubation for 8min. yields only one strongly radioactive zone. Initially this appeared strong evidence that albumin was unevenly labelled after incubation of the microsome fraction for short periods of time. Such an interpretation, however, was weakened by the subsequent finding that free methionine sulphoxide (methionine is oxidized to its sulphoxide in these experiments by treatment of albumin with performic acid) itself runs in exactly the same position as the single radioactive zone in the 8min. albumin sample. Unfortunately, after the 2 months' exposure to X-ray film required to produce darkening of the film in these experiments there was insufficient radioactivity remaining on the paper to study this possibility. Moreover, the amount of ninhydrin-positive material on the paper was too small to allow direct chemical detection of methionine. Though it seems unlikely that free methionine would remain attached to albumin throughout its rigorous isolation procedure the exact identity of the strong radioactive band in the 8min. albumin sample remains in doubt.

Table 2. *Specific activities and yields of albumin isolated after incubating the microsome fraction with* [^{35}S]*methionine and* [^{14}C]*isoleucine*

The microsome fraction from 40g. of regenerating rat liver was incubated with a total of 2800μc of [^{35}S]methionine (specific activity 430mc/m-mole) or with 30μc of [^{14}C]isoleucine (specific activity 246mc/m-mole). The microsome fraction was recovered by centrifuging at 104000*g* for 60min. and albumin extracted as detailed in the Materials and Methods section.

Labelled amino acid used	Time of incubation (min.)	Specific activity (counts/min./mg.)	Yield (μg.)
[^{35}S]Methionine	30	19600	360
[^{35}S]Methionine	8	3600	210
[^{14}C]Isoleucine	30	9000	234
[^{14}C]Isoleucine	5	2300	248
[^{14}C]Isoleucine	3·5	340	584

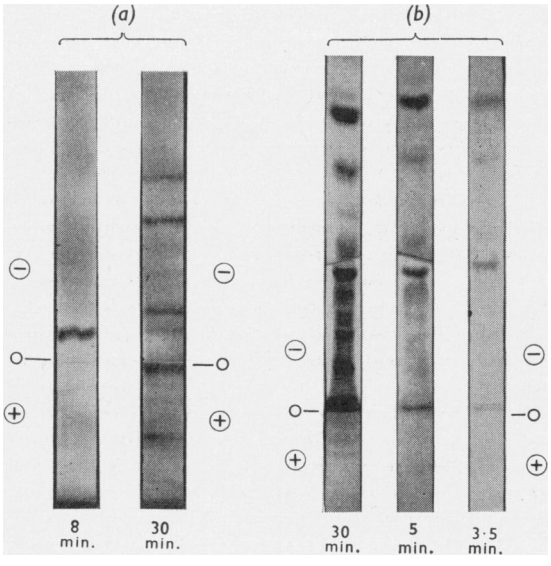

Fig. 3. Radioautographs of tryptic peptides from albumin isolated after incubating the microsome fraction with (*a*) [^{35}S]methionine and (*b*) [^{14}C]isoleucine for the different times indicated. The time of exposure of the paper to X-ray film was 2 months in (*a*) and 4 months in (*b*). Specific activities and amounts of albumin present are given in Table 2. Electrophoresis was carried out at 40v/cm. for 90min. in pyridine–acetate buffer, pH3·5.

The radioautographs from albumin labelled with [^{14}C]isoleucine show that after 30min. incubation there are 11 radioactive zones present. Incubation times of 5 and 3·5min., however, yield only six and three radioactive zones respectively. This is exactly in accord with the scheme depicted in Fig. 1, although the possibility remained that all the radioactive bands present in the 30min. sample were in fact present in the 3·5min. sample but at a greatly reduced radioactivity. In this case only the most intense bands would become visible and the weaker bands, though present, would escape detection. The observed differences of intensity of blackening in the zones in the 30min. sample suggest a variation in the specific radioactivity of the [^{14}C]isoleucine in the peptides, although the presence of more than one residue of [^{14}C]isoleucine in any given zone cannot be excluded.

Fate of methionine and isoleucine during the incorporation process. It was important to demonstrate that the radioactivity detected in the radioautographs was due to methionine and isoleucine in the two experiments and not to any other amino acids. This is particularly so for methionine, which might be converted into cysteine. Since the amount of protein handled in these experiments was too small to allow direct identification of amino acids on the 'fingerprints' the following approach was adopted. After incubation of the microsome fraction with cell sap, a source of energy and the

radioactive amino acids, a sample of the cell sap was removed and deproteinized by heating at 90° for 30min. Amino acids were isolated from the protein-free supernatant by adsorption on to Zeo-Karb 225 (H^+ form) columns and elution with N-ammonia solution. A sample of the eluate was examined by paper chromatography, with butan-1-ol–acetic acid–water (50:11:25, by vol.) as solvent for the methionine experiment and 2-methylbutan-2-ol saturated with water (Work, 1949) for the isoleucine experiments. After chromatography the papers were dried and examined for radioactivity by radioautography as for the albumin peptides. Areas of paper containing radioactivity were cut from the papers and counted directly in the Nuclear–Chicago gas-flow counter.

Cell sap from the methionine experiment revealed that not more than 5% of the total radioactivity present on the paper was due to cysteine, the remainder being accounted for solely as methionine. It may be concluded that the bulk of radioactivity in peptides from albumin labelled with [^{35}S]-methionine is due to methionine itself, but, since the cysteine content of albumin is some 5–6 times that of methionine it is probable that some of the activity detected is due to cysteine. This might especially be so where more than one cysteine residue is present in a given peptide and could account for the appearance of faintly radioactive zones, particularly those migrating towards the anode (cysteine is converted into cysteic acid by the performic acid treatment).

Cell sap from the isoleucine experiments revealed that all the radioactivity was present as isoleucine. Likewise experiments with [^{14}C]leucine (see below) demonstrated that this amino acid is not converted into other amino acids during incubation of microsomes with cell sap and an energy source.

Quantitative studies on the distribution of radioactivity in albumin. Though the radioautographic studies provide some evidence for the sequential synthesis of albumin the results were of a qualitative nature and could not be regarded as conclusive. It therefore became apparent that a direct measurement of the specific radioactivity of the labelled amino acid in each peptide was required for conclusive results. The following method involving the use of the two isotopes, ^{3}H and ^{14}C, as developed by Dintzis (1961) in his studies of haemoglobin, therefore was adopted.

Samples of albumin were isolated after incubating the microsomes for different periods of time with [^{14}C]leucine. Each of these albumin samples was then mixed with a sample of albumin that was evenly labelled throughout with [^{3}H]leucine. The latter sample was isolated from rat serum by the method of Debro *et al.* (1957) 3hr. after intravenous injection of 1mc of DL-[4,5-$^3H_2$]leucine (specific activity 5000mc/m-mole) into the animal. The mixed samples were then subjected to oxidation with performic acid and digestion with trypsin, and the resulting peptides were separated in two dimensions. The ^{14}C/^{3}H ratio in each peptide provides a measure of the specific activity of ^{14}C in that peptide. This measurement is independent of the yield of any given peptide and, more important, independent of the actual number of leucine residues in a peptide.

'Fingerprinting' of albumin. The amino acid composition of albumin showed that a maximum of 54 tryptic peptides could contain leucine. To separate such a complex mixture of peptides a two-dimensional 'fingerprinting' technique involving electrophoresis in one dimension followed by chromatography in a second dimension was used. Electrophoresis was in pyridine–acetate buffer, pH 3·5, followed by chromatography in butan-1-ol–acetic acid–water (3:1:1, by vol.). A diagram of a typical 'fingerprint' is shown in Fig. 4. Of the 74 tryptic peptides expected from albumin on the basis of its lysine and arginine content (Table 1) only some 45–47 peptides were detected with the ninhydrin reagent. While this method therefore does not account for the maximum theoretical number of tryptic peptides it was considered adequate for the present studies, especially in view of its excellent reproducibility from day to day. Fig. 4 also shows that albumin isolated 30min. after incubating the microsome with [^{14}C]leucine contains at least 21 peptides with significant ^{14}C activity as determined by direct counting. Fig. 4 also shows the presence at the origin of material that reacts with ninhydrin and that also contains radioactivity.

Quantitative distribution of activity in albumin labelled with [^{14}C]*leucine.* Albumin samples were isolated from the microsome fraction after incubation for 30 and 5min. with [^{14}C]leucine as detailed in Fig. 5 and the Materials and Methods section. Albumin was isolated also from the microsome fraction obtained from the liver of a rat 30min. after it had received an intravenous injection of [^{14}C]leucine. Each of these samples was mixed with a sample of albumin evenly labelled with [4,5-$^3H_2$]leucine as described in Fig. 5 and the mixed samples were subjected to the two-dimensional 'fingerprinting' procedure described above. The ^{14}C/^{3}H ratios in each peptide were arranged to give the results shown in Fig. 5. The peptides from albumin isolated directly from the microsome fraction after injecting the labelled leucine into the animal have nearly constant specific activity. In contrast, albumin isolated after incubating the isolated microsome fraction for 30min. has a marked gradient of activity within the molecule. Short incubation times, however, result in only a few

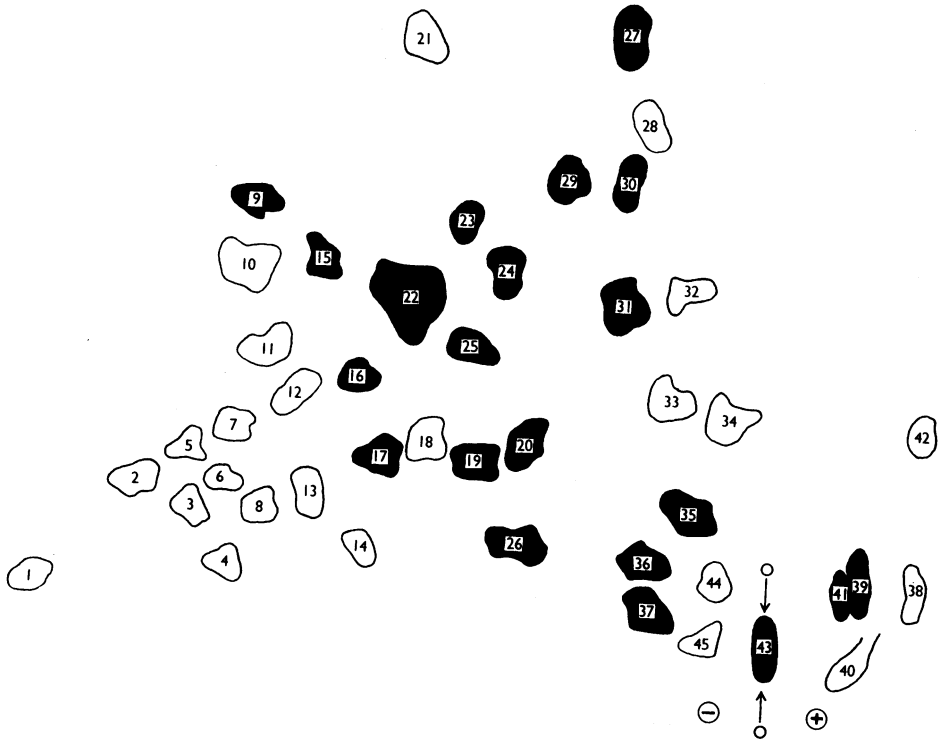

Fig. 4. 'Fingerprints' of tryptic peptides from rat serum albumin. Experimental details are given in the Materials and Methods section. All spots shown gave a positive reaction with ninhydrin. Numbers within each peptide are used in Fig. 5. Shaded areas represent those peptides with significant radioactivity, as measured in Fig. 5.

peptides being labelled in the protein, and the peptides in this case correspond to those peptides that have the highest specific activity in the 30 min. albumin sample. These results are entirely consistent with the scheme of sequential synthesis depicted in Fig. 1. The peptides in Fig. 5 were arranged according to their positions in the 'fingerprints' (Fig. 4) in the sequence: 22, 9, 29, 24, 19, 31, 37, 23, 35, 30, 15, 41, 25, 17, 39, 16, 36, 26, 20, 27.

Specific activity of leucine from near the C-*terminus of albumin.* If the sequence of growth of albumin is from the *N*-terminus towards the *C*-terminus, as in other proteins studied (see the introduction section), then it may be predicted that leucine residues from near the *C*-terminus of albumin would have higher specific activities than leucine residues from near the *N*-terminus. It has been reported by Jungblut & Turba (1963) that treatment of albumin with carboxypeptidase A releases two residues of alanine and one residue of leucine from the protein and that the *C*-terminus of albumin is probably -Ala-Leu-Ala. Analysis of the amino acids released from the *C*-terminus of albumin by treatment with carboxypeptidase A–DFP as described in the Materials and Methods section showed that one residue each of alanine and leucine were released under the present conditions. It was therefore possible to compare the specific activity of leucine released by carboxypeptidase A with the specific activity of the remaining leucine residues in albumin.

Albumin (specific activity 104 500 disintegrations/min./mg.) was isolated with the aid of carrier serum albumin 30 min. after incubating the isolated microsome fraction with 200 μc of L-[U-^{14}C]leucine (specific activity 198 mc/m-mole). A sample (201 μg.) of this albumin was mixed with 3·0 mg. of albumin (specific activity 44 100 disintegrations/min./mg.) isolated from serum 3 hr. after injecting 1 mc of DL-[4,5-$^{3}H_2$]leucine (specific activity 5000 mc/m-mole) into the animal. The mixed sample was digested with carboxypeptidase A–DFP and the ^{14}C/^{3}H ratio determined in the leucine released and in the residual protein as detailed in the Materials and Methods section. The ^{14}C/^{3}H ratio of the leucine released was 1·86 and that of the residual protein was 1·00.

Albumin (specific activity 21000 disintegrations/min./mg.) was also isolated directly from the microsome fraction with the aid of carrier serum albumin

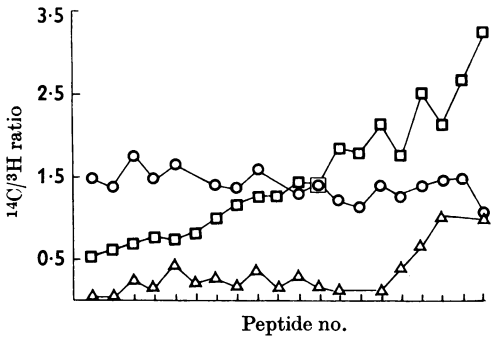

Fig. 5. ○, Albumin (specific activity 85000 disintegrations/min./mg.) was isolated from rat serum 30 min. after the injection of 60 μc of L-[^{14}C]leucine (specific activity 23·4 mc/m-mole). □, Albumin (specific activity 54500 disintegrations/min./mg.) was isolated from the microsome fraction 30 min. after incubating with 40 μc of L-[U-^{14}C]leucine (specific activity 47 mc/m-mole). △, Albumin (specific activity 12500 disintegrations/min./mg.) was isolated from the microsome fraction 7·5 min. after incubating with 40 μc of L-[U-^{14}C]leucine (specific activity 47 mc/m-mole). Then 480, 396 and 189 μg. of albumin from ○, □ and △ respectively were each mixed with 2·13 mg. of albumin (specific activity 44108 disintegrations/min./mg.) isolated from rat serum 3 hr. after injecting 1·0 mc of DL-[4,5-$^{3}H_2$]leucine (specific activity 5000 mc/m-mole) into the animal. Mixed samples were subjected to performic acid oxidation and digestion with trypsin, the peptides were separated by electrophoresis and chromatography, and $^{14}C/^{3}H$ ratios were determined for each peptide as described in the Materials and Methods section. Peptides were arranged arbitrarily in order of increasing ratio according to the numbering in Fig. 4 in the sequence 22, 9, 29, 24, 19, 31, 37, 23, 35, 30, 15, 41, 25, 17, 39, 16, 36, 26, 20, 27. The following peptides were not measured: 31 and 30 in ○, and 17 and 20 in △.

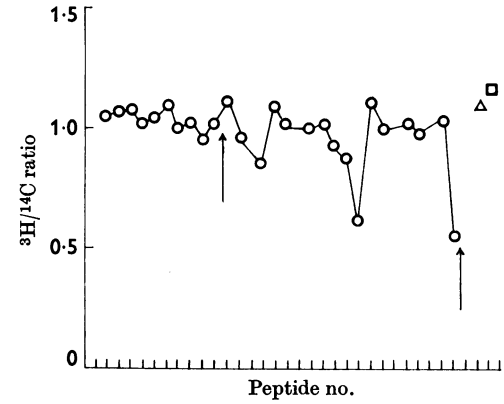

Fig. 6. Effect of carboxypeptidase A–DFP on the distribution of radioactivity in serum albumin evenly labelled with [^{3}H]leucine. Albumin (specific activity 577950 disintegrations/min./mg.) was isolated from rat serum 1 hr. after injecting 5 mc of DL-[4,5-$^{3}H_2$]leucine (specific activity 10900 mc/m-mole) into the animal; the protein was digested with 1% of its weight of carboxypeptidase A–DFP as detailed in the Materials and Methods section. A 0·6 mg. sample of the protein remaining after digestion and removal of free amino acids was mixed with 2·17 mg. of albumin (specific activity 33000 disintegrations/min./mg.) isolated directly from the microsome fraction, with the aid of carrier serum albumin, 1 hr. after the injection of 150 μc of L-[U-^{14}C]leucine (specific activity 160 mc/m-mole) into the animal. The mixed sample was subjected to oxidation with performic acid and digestion with trypsin, the peptides were separated by electrophoresis and chromatography and the $^{3}H/^{14}C$ ratio was determined for each peptide. Peptides numbered as in Fig. 4 were arranged in the sequence 10, 13, 7, 14, 18, 23, 21, 32, 22, 9, 29, 24, 19, 31, 37, 23, 35, 30, 15, 41, 25, 17, 39, 16, 36, 26, 20, 27. Peptides 29, 37, 39 and 26 were not measured. The arrows indicate that part of the sequence which is used in Fig. 5. [^{3}H]Albumin and [^{14}C]albumin as above were also mixed before treatment with carboxypeptidase A–DFP and the amino acids released separated from the residual protein (see the Materials and Methods section). Ratios for the amino acids released (△) and the residual protein (□) are also shown.

1 hr. after injecting 150 μc of L-[U-^{14}C]leucine (specific activity 160 mc/m-mole) into the animal. A sample (2·9 mg.) of this albumin was mixed with 0·6 mg. of albumin (specific activity 577950 disintegrations/min./mg.) isolated from rat serum 1 hr. after injecting 5 mc of DL-[4,5-$^{3}H_2$]leucine (specific activity 10900 mc/m-mole) into the animal. In this experiment the $^{14}C/^{3}H$ ratio in the leucine released by carboxypeptidase A–DFP was 1·06 and that in the residual protein was 1·00.

This experiment demonstrates that the *C*-terminal leucine from albumin isolated after incubating the isolated microsome fraction has a specific activity nearly twice the mean specific activity of the remaining leucine residues in the protein. For albumin isolated directly from the microsome fraction 60 min. after injecting labelled leucine the *C*-terminal leucine has essentially the same specific activity as the remaining leucine residues in albumin. These results confirm that albumin isolated from the microsome fraction 60 min. after injecting labelled leucine is evenly labelled, whereas albumin isolated 30 min. after incubating the isolated fraction is unevenly labelled. This is evidence, moreover, for a sequential synthesis of albumin, the direction of growth of the molecule being towards the *C*-terminus.

Identification of the C*-terminus peptide in albumin.* The above evidence that the direction of growth of albumin is towards the *C*-terminus leads to the prediction that the peptide with the highest specific activity in Fig. 5 is probably derived from the *C*-terminus of the protein. This prediction was tested as follows. Albumin evenly labelled with [^{3}H]leucine was isolated from rat serum 60 min. after the injection of [^{3}H]leucine into the animal (Fig. 6). Albumin evenly labelled with [^{14}C]leucine was also isolated directly from the microsome

fraction 60min. after injecting [^{14}C]leucine into the animal (Fig. 6). The [^{3}H]albumin was treated with carboxypeptidase A–DFP, and the protein remaining after digestion was isolated and mixed with a sample of the untreated [^{14}C]albumin. The mixed sample was submitted to the 'fingerprinting' procedure and the ^{3}H/^{14}C ratio measured in each peptide. Since the [^{14}C]albumin was present in excess (Fig. 6) the full complement of peptides was detected with ninhydrin, and it was expected that all these peptides should have a constant ^{3}H/^{14}C ratio except that peptide which is derived from the *C*-terminus. This latter peptide should have a decreased ^{3}H/^{14}C ratio since only [^{3}H]albumin was treated with carboxypeptidase A–DFP and therefore only [^{3}H]leucine and not [^{14}C]leucine will have been removed from the peptide. In the results shown in Fig. 6 the peptides have been arranged in exactly the same sequence as depicted in Fig. 5: of some 25 peptides tested in this experiment only two show a substantial deviation from a nearly constant ratio. Of these peptides peptide no. 27 is placed at the end of the sequence and is almost certainly the *C*-terminus peptide of albumin. Peptide no. 41, which also shows a decreased ratio, is some distance from the end of the sequence, and in view of this seems unlikely to be a true *C*-terminus peptide. This result in conjunction with Fig. 5 may be taken as further evidence for the direction of growth of albumin towards the *C*-terminus.

Incorporation of radioactivity into the N-*terminus of albumin.* The foregoing results provide evidence that the synthesis of albumin in the isolated microsome fraction is sequential and that the direction of growth is towards the *C*-terminus. From this it could be predicted that the *N*-terminus of the protein should have a lower specific activity than any other residue in the protein. In view of the marked gradient of activity in Fig. 5 it was decided, however, to determine whether any radioactivity at all could be incorporated into the *N*-terminus of albumin. The *N*-terminus of rat albumin as determined by the DNP method has been reported by a number of authors to be glutamic acid (Peters, 1962*a*; Jungblut & Turba, 1963; Busch, Fujiwara & Firszt, 1961; the present paper).

The isolated microsome fraction from 40g. of regenerating rat liver was incubated for 30min. with a total of 80μc of L-[U-^{14}C]glutamic acid (specific activity 38mc/m-mole) and microsomal albumin isolated. Albumin with a specific activity of 4200counts/min./mg. was isolated. Since glutamic acid may be converted into other amino acids during incubation with cell sap the radioactivity in this sample of albumin would not be expected to be confined solely to glutamic acid. Care was taken to determine the specific radioactivity of the *N*-terminal glutamic acid residue and the remaining glutamic acid residues in albumin under as nearly identical conditions as possible. DNP-glutamic acid from the *N*-terminus and DNP-glutamic acid from the interior of the protein were isolated as detailed in the Materials and Methods section. Sufficient of the internal DNP-glutamic acid was taken to give approximately the same number of counts/min. as the *N*-terminal DNP-glutamic acid residue. The use of a Nuclear–Chicago low-background counter (see the Materials and Methods section) made possible the accurate determination of count rates down to less than 1count/min. above background.

The specific activity of the *N*-terminal DNP-glutamic acid residue was 44counts/min./μmole, and the mean specific activity of the remaining glutamic acid residue in albumin was 149counts/min./μmole. It is difficult to be certain of the exact interpretation of these values since DNP-glutamic acid from the interior of the protein will be contaminated with DNP-glutamic acid derived from glutamine during hydrolysis of the DNP-protein. It was confirmed in the present experiment that radioactivity from glutamic acid is incorporated into glutamine during incubation of cell sap with microsomes so that the absolute specific activity of the internal glutamic acid is obscured by the addition of an unknown amount of [^{14}C]glutamine of unknown specific activity. The specific activity of the [^{14}C]glutamine would be expected to be considerably less than that of the [^{14}C]glutamic acid present in cell sap and certainly it could never exceed it. It is probable therefore that the value quoted here for the specific activity of the internal glutamic residues is a minimum. Attempts to eliminate these difficulties by enzymic hydrolysis of DNP-albumin by using trypsin, chymotrypsin, pepsin, Nagarse or Pronase were not successful.

Despite the uncertainties inherent in the quantitative aspects of this experiment there is no doubt that radioactivity is incorporated into the *N*-terminus of albumin, thus indicating that some degree of synthesis of complete chains has occurred during incubation of the microsomes. Moreover, such activity is considerably less than that of the mean specific activity of the combined glutamic acid and glutamine residues in the protein, a finding that is consistent with sequential synthesis from the *N*-terminus.

DISCUSSION

Campbell *et al.* (1960) demonstrated that, in the presence of cell sap and a source of energy, the isolated microsome fraction from rat liver could incorporate a radioactive amino acid into a protein with the immunological, solubility and electropho-

retic characteristics of serum albumin. Subsequent studies (Campbell & Kernot, 1962) showed that [^{14}C]leucine was incorporated into peptide linkage in albumin under these conditions. The present work has confirmed the latter finding (Figs. 5 and 6) and has shown also that [^{14}C]isoleucine, [^{35}S]-methionine and [^{14}C]glutamic acid are incorporated into peptide linkage in albumin in the subcellular system (Fig. 3). These results lead to the conclusion that at least some degree of synthesis of albumin has taken place in the microsome system. Two major aspects of the work require discussion: first, the evidence that it provides for the mode of assembly of amino acids in the polypeptide chain; secondly, the extent to which the isolated microsome fraction is capable of assembling the complete chain.

Both the radioautographic experiments with [^{14}C]isoleucine and [^{35}S]methionine and the quantitative experiments with [^{14}C]leucine (Figs. 3 and 5) are entirely consistent with the scheme of sequential synthesis of albumin depicted in Fig. 1. Further evidence for sequential synthesis is found in the experiments with carboxypeptidase A related to the direction of growth of the molecule (Fig. 6). There seems little doubt therefore that a mechanism of sequential synthesis of albumin is taking place in the isolated microsome fraction. Such a mechanism satisfactorily explains the apparent differences in specific activities in radioactive zones in the radioautographs (Fig. 3) and the marked gradient of activity in Fig. 5. Apparent differences in specific activities in radioactive peptide zones from tryptic peptides of albumin labelled with [^{14}C]leucine were also demonstrated by Campbell & Kernot (1962). The interpretation of the previous experiments is clearer now that it has been demonstrated that under the conditions employed *in vivo* the specific radioactivity of the leucine would be uniform throughout the mixture of peptides.

Several factors have a bearing on the extent to which the present results reflect a synthesis of the complete molecule of serum albumin in the isolated microsome fraction. Total synthesis of the chain in the presence of a ^{14}C-labelled amino acid would result in all the residues containing the particular amino acid becoming radioactive. Fig. 3 reveals that all six of the expected radioactive peptides are obtained from albumin labelled with [^{35}S]-methionine, whereas only about 11 radioactive peptides out of the possible 14 are obtained from albumin labelled with [^{14}C]isoleucine. In the latter experiment the single-dimension electrophoresis may not have resolved completely all the radioactive peptides and it is possible that more than one is present in any given radioactive zone. Conversely, a single peptide could also contain more than one isoleucine residue. The latter consideration also applies to the experiment with [^{14}C]leucine (Fig. 5) where out of a maximum of 54 labelled peptides at least 21 could be detected from a radioautograph. In this connexion the material remaining at the origin in the 'fingerprints', whether in one or two dimensions, is important. This material gave a positive reaction with ninhydrin and always contained radioactivity when derived from albumin isolated after incubating the isolated microsomes for 30min. The material was not digested by further treatment with trypsin but was at least partially degraded by treatment with chymotrypsin (J. R. Sargent & P. N. Campbell, unpublished work) and could represent a tryptic 'core' rich in hydrophobic groups similar to that found in haemoglobin (Hunt & Ingram, 1958).

A further reason why the maximum number of radioactive peptides was not detected in these experiments stems from the gradient of radioactivity present in all albumin samples isolated from experiments *in vitro*. The presence of such a gradient demands in effect the measurement of radioactivity ranging from relatively high to very low levels. Radioautography and scintillation counting would not detect radioactivity in the peptides that contained only small amounts of radioactive amino acid. The failure to account for radioactivity in all the expected peptides from albumin is not therefore inconsistent with the presence of some activity in all peptides. Indeed, one would expect this to be the case from the experiments with [^{14}C]glutamic acid, in which very sensitive methods were used to determine radioactivity. The method of isolation of albumin from the microsome pellet was sufficiently rigorous to ensure that only virtually complete chains of albumin would have survived the process. The fact that the *N*-terminal glutamic acid was radioactive and that the direction of growth was from the *N*-terminus to the *C*-terminus must imply the synthesis of the complete chain. The gradient of activity in Fig. 5, however, demonstrates that most of the incorporation of amino acid into albumin under our conditions is due to the completion of pre-existing chains.

The results in Figs. 5 and 6 provide evidence that albumin is evenly labelled when isolated from serum either 1 or 3hr. after injecting labelled amino acid into the animal, or when isolated directly from the microsome fraction either 1hr. or 30min. after injecting labelled amino acid. These results do not substantiate the report by Fried & Neet (1961) of uneven labelling in rabbit albumin after the injection of [^{14}C]lysine. The latter result was interpreted as being inconsistent with the sequential synthesis of albumin; Fried (1964) has obtained similar results by the use of [^{14}C]lysine in isolated perfused rat liver. In contrast, using the same system with [^{14}C]leucine, Jungblut (1963) obtained

evidence for sequential synthesis of the protein with direction of growth towards the *C*-terminus.

The findings that leucine from the *C*-terminus of albumin has higher specific activity than the remaining leucine residues in the protein and that the peptide with the highest specific activity in Fig. 5 is probably derived from the *C*-terminus (Fig. 6) strongly suggest that the direction of synthesis of albumin is from the *N*-terminus towards the *C*-terminus. This is fully borne out by the finding that the *N*-terminal glutamic acid has lower specific activity than the remaining glutamic acid residues in albumin. As first pointed out by Dintzis (1961), a study of sequential synthesis of a protein can provide valuable information about the amino acid sequence of the protein. In this light one is tempted to state that the sequence in which the peptides have been arranged in Fig. 5 represents the sequence of these peptides in albumin from the *N*-terminus towards the *C*-terminus. Such a statement would, however, be rash until all the tryptic peptides in albumin have been accounted for, since, although the 'core' itself is presumably a large peptide that can be fitted into such a sequence, the presence of only 45–47 peptides means that the possibility of incomplete resolution of peptides and possible overlapping cannot be ruled out. It is difficult, moreover, to be certain of the exact sequence of the peptides in Fig. 5 owing to the limited amounts of radioactivity incorporated into albumin and the consequently limited amounts of radioactivity present in each peptide. On the basis of the present experiments with albumin, it is considered that an approach to studying the sequence of albumin based on sequential synthesis would best be applied in the first instance to fairly large fragments of the molecule obtained by specific cleavage of, e.g., methionine bonds with cyanogen bromide (Gross & Witkop, 1962). Such cleavage, however, has not been successful as yet with albumin. Results recently obtained with limited proteolysis by pepsin appear promising (Weber & Young, 1964).

The value and perhaps the limitations of the present work with respect to sequence studies is illustrated in the experiment designed to identify the *C*-terminal peptide in albumin (Fig. 6). The finding that peptide no. 27, the peptide with the highest specific activity in Fig. 5, is affected by treatment of the intact protein with carboxypeptidase A–DFP is good evidence that this peptide is, in fact, at the *C*-terminus. Peptide no. 41, however, also shows a significant decrease in ^3H specific activity in this experiment, and it could be inferred that it also was derived from the *C*-terminus. The latter possibility is unlikely in view of the placing of this peptide away from the end of the sequence in Fig. 5, and, moreover, such an interpretation would demand that albumin consisted of two chains with identical *N*- and *C*-termini. There is no doubt that a single *N*-terminus and a single *C*-terminus are present in albumin (Peters, 1962*a*; Jungblut & Turba, 1963; Busch *et al.* 1961), but separation of albumin into two separate chains or sub-units has not been reported. These factors strongly suggest that the protein contains a single large polypeptide chain. Such an interpretation, however, must be viewed in light of the suggestion by Ochoa (1963) that most proteins are formed by the aggregation of smaller sub-units, so that the presence of two *C*-termini in albumin cannot be definitely ruled out. One possible explanation of the behaviour of peptide no. 41 in Fig. 6 is that the isolation procedure for albumin in this experiment, which involves heating with trichloroacetic acid at 90° for 20min. to denature the protein and brief treatment with alkali to render the denatured protein soluble, has ruptured a bond in the molecule that is particularly sensitive to hydrolysis. Such an effect would result in the appearance of two *C*-termini before the treatment with carboxypeptidase A.

In conclusion, the implications of the results on serum albumin may be considered in the light of present concepts of the mechanism of protein biosynthesis. The demonstration of the sequential assembly of the polypeptide chain of serum albumin is in accord with the results obtained for haemoglobin (Bishop *et al.* 1960; Dintzis, 1961; Naughton & Dintzis, 1962), for bacterial amylase (Yoshida & Tobita, 1960), for lysozyme (Canfield & Anfinsen, 1963) and for ribonuclease (Luck & Barry, 1964). The present results differ from the others only in that albumin is a much larger protein containing 575 amino acids in a single chain.

If proteins are synthesized in association with a strand of messenger RNA then it must be presumed that the isolated microsome fraction from rat liver contains such strands. We have demonstrated that synthesis of the complete polypeptide can take place, but more often synthesis involves the completion of chains that pre-exist at the time of isolation. Three nucleotides are required to code for each amino acid (Leder & Nirenberg, 1964), so that for a polypeptide chain of 575 amino acids one requires a molecule of messenger RNA containing 1725 nucleotides. Slayter, Warner, Rich & Hall (1963) have shown that on average the distance between the centres of the ribosomes spaced along a molecule of messenger RNA is 90 nucleotides, which represents the code for 30 amino acids. From this it follows that the polysome for the synthesis of albumin should contain 19 ribosomes. On this basis the isolated microsome fraction should therefore contain 19 unit polysomes for the synthesis of albumin.

The failure of the subcellular system to synthesize more than a small proportion of complete chains of serum albumin may be due to the paucity of large polysomes as a result of damage during the isolation process or to a metabolic failure of the system. The latter explanation seems the more likely, for it is not only albumin synthesis that is short-lived but the incorporation of amino acids in general. It would therefore be wrong to conclude that the complete polysome requirement for albumin synthesis is not present in the microsome fraction. Nevertheless, since much of the synthetic capacity is concerned with the completion of chains that were initially not more than half complete, it is possible only to predict that polysomes containing eight or nine ribosomes would be of general occurrence.

In previous experiments it has proved difficult to demonstrate the synthesis of albumin by ribonucleoprotein preparations derived from the microsome fraction after the removal of the membrane by deoxycholate in spite of the fact that such preparations readily incorporated ^{14}C-labelled amino acid into uncharacterized protein (Decken & Campbell, 1962). This could have been due to the lack of polysomes in such preparations. The polysome hypothesis predicts that monomeric ribosome units attached to a short chain of messenger RNA will be able to incorporate amino acids into a peptide, but only those units that have been derived from the end of the messenger RNA at which protein synthesis is terminated will be able to complete the synthesis of the polypeptide chain of a protein such as serum albumin. Although it has been shown that deoxycholate itself does not destroy polysomes (Warner, Knopf & Rich, 1963), it is possible that the phospholipid membrane of the rough-surfaced endoplasmic reticulum stabilizes the structure as shown by Schlessinger (1963) in *Bacillus megaterium*. These ideas have been developed in greater detail by Campbell (1965) and were briefly discussed by Campbell & Sargent (1964).

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