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Characterization and Biosynthesis of Cytochrome b_5 in Rat Liver Microsomes

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1. Cytochrome b_5 is released from rat liver microsomes by both proteolytic enzymes and by treatments that disrupt phospholipids. Cytochrome *P*-420 is only released to a marked extent by treatments that disrupt phospholipids. 2. Cytochrome b_5 was isolated in a pure state from both the rough and smooth fractions of rat liver microsomes after treatment with trypsin, and was shown to contain two cytochrome components with identical spectral properties. 3. Amino acid analyses of the two components are presented, together with peptide 'fingerprint' patterns of tryptic digests of the two components. 4. Studies based on the direct isolation of cytochrome b_5 after administration of a single dose of radioactive amino acid to rats demonstrate that the cytochrome is synthesized initially in the rough fraction of microsomes and only subsequently appears in the smooth fraction. 5. Isolated rat liver microsomes are capable of incorporating radioactive amino acids into cytochrome b_5 under standard conditions. 6. Under these conditions the amino acid is incorporated into peptide linkage in the cytochrome.

Recent studies concerned with the biogenesis of membranes of the endoplasmic reticulum of liver have demonstrated that certain constitutive proteins of the membranes are synthesized initially in the rough-surfaced fraction of the reticulum, and only subsequently appear in the smooth-surfaced fraction (Ernster & Orrenius, 1965; Dallner, Siekevitz & Palade, 1966*a*,*b*). The present work relates to the biogenesis of membranes in that it is concerned with the biosynthesis of a specific protein component of the endoplasmic reticulum, cytochrome b_5. This haemoprotein has been isolated in a pure state from calf liver microsomes (Strittmatter & Vellick, 1956; Strittmatter, 1960; Strittmatter & Ozols, 1966*a*) and from pig liver microsomes (Garfinkel, 1957*a*,*b*; Poltoratsky-Bois & Chaix, 1964), and has been thoroughly characterized in recent years (Strittmatter, 1960; Strittmatter & Ozols, 1966*a*,*b*; Ozols & Strittmatter, 1967).

The present work demonstrates that cytochrome b_5 is synthesized initially in the rough fraction of rat liver microsomes, and only subsequently appears in the smooth fraction. Isolated microsomes synthesize cytochrome b_5 to at least a limited extent. As a preliminary to these biosynthetic studies, some of the properties of cytochrome b_5 from rat liver were studied. The results presented below have already been communicated (Sargent & Vadlamudi, 1967; Vadlamudi & Sargent, 1967) in a preliminary form.

MATERIALS AND METHODS

Radiochemicals. The following radioactive amino acids were purchased from The Radiochemical Centre (Amersham, Bucks.): L-[4,5-^{3}H]leucine (35100mc/m-mole), L-[U-^{14}C]leucine (155mc/m-mole) and L-[U-^{14}C]lysine (various specific activities).

Animals. Animals used in these studies were Wistar-strain male albino rats purchased from Tuck and Sons Ltd. (Rayleigh, Essex). Animals weighed 160–250g. and were starved for 16hr. before being killed.

Administration of radioactive amino acids to rats. A solution of 0·3ml. of radioactive amino acid in 0·9% NaCl was administered to rats under light ether anaesthesia by way of the penial vein.

Isolation of microsomes. When microsomes were to be isolated from livers of rats that had received an injection of radioactive amino acid, the animals were lightly anaesthetized with ether and the livers perfused *in situ* with chilled 0·9% NaCl. The solution was administered by way of the portal vein after the superior vena cava had been cut. The livers paled within seconds of starting the perfusion, and approx. 50ml. of 0·9% NaCl was perfused through each liver. All subsequent steps were performed at 2°. Portions (4g.) of liver were chopped finely and homogenized with 10ml. lots of 1·15% (w/v) KCl in a mechanically driven all-glass homogenizer of the Potter type with a clearance of approx. 0·01in. The homogenate was centrifuged at

10000$g_{av.}$ for 10min. and the resulting supernatant centrifuged at 105000$g_{av.}$ for 60min. to sediment the microsome fraction. This microsome fraction was suspended in 1·15% KCl with homogenization, and the microsomes were sedimented once more at 105000$g_{av.}$ for 60min. The microsomes were finally suspended in chilled 0·1M-tris–HCl buffer, pH 8·5 (at room temp.), to give a protein concentration of 5–10mg./ml. Spectroscopic examination of either whole microsomes or subfractions of microsomes prepared in this way showed that no detectable haemoglobin was present.

When microsomes were to be prepared for amino acid incorporation *in vitro*, conventional methods were used as described by Sargent & Campbell (1965). In this case the livers were not perfused.

Isolation of rough and smooth fractions of microsomes. Rough and smooth fractions of microsomes were isolated by centrifuging through a discontinuous sucrose gradient in a medium containing CsCl, exactly as described by Dallner (1963). The rough fraction obtained as a pellet was suspended in 0·1M-tris–HCl buffer, pH 8·5, to give a final protein concentration of about 5–10mg./ml. The smooth fraction separated into two layers at the interphase of the sucrose layers. In all experiments these two fractions were combined, sedimented and suspended in 0·1M-tris–HCl buffer, pH 8·5, to give a protein concentration of 5–10mg./ml.

Incubation of isolated microsomes. For amino acid incorporation *in vitro*, sedimented microsomes from 4g. of liver were suspended with homogenization in 1·5ml. of a medium containing 35mM-tris–HCl buffer, pH 7·8 (at room temperature), 25mM-KCl, 10mM-$MgCl_2$ and 0·15M-sucrose (medium A). Cell sap was passed through a column of Sephadex G-25 equilibrated with medium A. The standard amino acid-incorporation medium contained 0·4ml. of the above microsomes, 0·2ml. of cell sap, 15μmoles of phosphoenolpyruvate, 2μmoles of ATP, 0·25μmole of GTP and 50μg. of pyruvate kinase in a total volume of 1·0ml. The radioactive amino acids added are detailed in individual experiments. The suspension was incubated at 37° for 30min. When total protein was to be isolated the reaction was terminated by adding trichloroacetic acid (final concn. 5%, w/v). When cytochrome b_5 was to be isolated the reaction was stopped by rapid chilling and at the same time the unlabelled counterpart of the radioactive amino acid used was added to a concentration of 1mM. The microsomes were recovered by centrifuging at 105000$g_{av.}$ for 60min., washed once with 1·15% KCl and suspended in 0·1M-tris–HCl buffer, pH 8·5, to give a protein concentration of 5–10mg./ml.

Treatment of microsomes with various agents. In one experiment the effects of various agents on the release of cytochromes from microsomes were studied. Microsomes (15mg. of protein/ml. in 0·1M-tris–HCl buffer, pH 8·5) were treated with various enzyme preparations at 2° for 48hr. In each case the enzymic protein was present at 1% (w/w) of the total microsomal protein. Where detergents were used these were added to a final concentration of 1% (w/v) and incubation was carried out at 2° for 48hr. Treatment with ultrasound was carried out with a Soniprobe type 1130A instrument (Daw and Co., London, W. 3) at various settings. After ultrasonic treatment the suspension was stored at −15° for 16hr. and thawed at 37°. In all cases suspensions were centrifuged at 105000$g_{av.}$ for 120min. and total protein, cytochrome b_5 and cytochrome *P*-420 were assayed in the supernatant as detailed below.

Isolation of cytochrome b_5 *from microsomes or subfractions of microsomes.* All fractions were suspended in 0·1M-tris–HCl buffer, pH 8·5, at a concentration of 5–10mg. of protein/ml. Solid trypsin equal to 1% (w/w) of the total protein present was added to the chilled suspension and the whole stirred gently at 2° for 18hr. The suspension was centrifuged at 105000$g_{av.}$ for 120min. to yield a tightly packed yellow pellet, overlaid with a loose fluffy red layer and a clear red supernatant. The latter was carefully decanted and cytochrome b_5 isolated essentially as described by Strittmatter (1960). In this method the supernatant was subjected to $(NH_4)_2SO_4$ fractionation at pH 8·5, the precipitates obtained at 40%, 60% and 80% saturation being discarded. The pH of the supernatant was finally adjusted to 4·0 to precipitate crude cytochrome b_5. The red precipitate was dissolved in a small volume of 0·05M-sodium phosphate buffer, pH 6·8, and passed through a column of Sephadex G-25 equilibrated with the same buffer. The red effluent was then adsorbed on a column of DEAE-cellulose (10cm. × 1·0cm.) equilibrated with 0·05M-sodium phosphate buffer, pH 6·8, and elution continued with the same buffer. A non-coloured contaminating protein was eluted almost immediately. Elution was continued for approx. 48hr., when the cytochrome zone had moved well down the column to separate into two red fractions. The top 2cm. of the DEAE-cellulose was removed with a Pasteur pipette to eliminate a second non-coloured contaminating protein, which was tightly adsorbed on the top of the column. When 'total' cytochrome b_5 was to be isolated the remaining column was eluted with M-NH_4HCO_3 and a single red fraction collected. When subfractions of cytochrome b_5 were to be isolated the upper zone of the DEAE-cellulose, containing the slower-running component (cytochrome b_5S), was removed with a Pasteur pipette and repacked on to a short column of DEAE-cellulose equilibrated with 0·05M-sodium phosphate buffer, pH 6·8. Both this column and the residual column containing the faster-running subfraction (cytochrome b_5F) were eluted with M-NH_4HCO_3. Solutions containing cytochrome were finally desalted by passage through a column of Sephadex G-25 equilibrated with mM-NH_4HCO_3 and freeze-drying.

Electrophoresis of cytochrome b_5. Starch-gel electrophoresis was carried out in 21·5mM-sodium borate buffer, pH 8·5, or 0·11M-sodium acetate buffer, pH 4·5, as described by Bodman (1960). Electrophoresis was also carried out on 1in. × 6¾in. strips of cellulose polyacetate (Sepraphore III, supplied by Gelman Instrument Co., Ann Arbor, Mich., U.S.A.) in either 0·11M-sodium acetate buffer, pH 4·5, or the glycerol–sodium barbiturate buffer, pH 8·6, described by Afonso (1961). Protein and haem were detected by staining with naphthalene black and *o*-dianisidine reagents respectively, as detailed by Bodman (1960).

Determination of cytochrome b_5. Cytochrome b_5 was determined quantitatively from its dithionite-reduced spectrum recorded in a Unicam SP. 800 spectrophotometer. An ϵ_{mM} value of 25·6cm.$^{-1}$ for the α-peak at 556mμ was used (Strittmatter & Velick, 1956).

Determination of cytochrome P-420. Cytochrome *P*-420 was determined by recording the CO + dithionite-reduced spectrum minus the dithionite-reduced spectrum in the

Unicam SP.800 instrument. An $\epsilon_{max.}$ value of 110 cm.$^{-1}$ between E_{420} and E_{490} was used (Omura & Sato, 1964*a*,*b*).

Removal of haem from cytochrome b_5. Haem was removed from the cytochrome essentially as detailed by Teale (1959). To a solution of the cytochrome at 2°, either in water or in NH_4HCO_3, was added 1 drop of 100% formic acid. The pH fell to 2–3. The solution was extracted four times with redistilled butan-2-one, the organic phase being removed in each case. Residual butan-2-one was removed from the aqueous phase under vacuum, and the apoprotein precipitated by adding trichloroacetic acid (final concn. 5%, w/v). The precipitated protein was washed with 5% trichloroacetic acid, acetone and finally ether, and air-dried.

Haem was recovered from the organic phases by washing the latter with water and evaporation to dryness under vacuum.

Digestion of apoprotein with trypsin. Apoprotein (500 μg.) prepared as above was dissolved in 0·5 ml. of 0·5 M-NH_4HCO_3, and trypsin (5 μg. in 5 μl. of water) was added. After incubation at 37° for 2 hr. a further 5 μg. of trypsin was added and digestion continued for a further 2 hr. The solution was freeze-dried.

Separation of tryptic peptides. Freeze-dried peptides were either dissolved in water or suspended in pyridine–acetate buffer, pH 6·5, and applied to layers of silica gel G (20 cm. × 20 cm.), precoated on to plastic (Kodak Chromagram sheet, type K 301R2, supplied by Kodak Ltd., Kirkby, Liverpool). These layers had previously been sprayed evenly with pyridine–acetate buffer, pH 6·5, laid over a glass plate (20 cm. × 20 cm.) and assembled in a Shandon Universal electrophoresis apparatus (type II, after Kohn) containing the same buffer. Contact was effected between the buffer in the tank and the thin layers by sheets of Whatman no. 3MM paper soaked in the buffer. Before application of peptides, electrophoresis was carried out at 310 v (10 mA) for at least 5 min. to ensure even wetness of the layer. Peptides (about 100–250 μg. in 2–5 μl. of solution) were applied some 7 cm. from the anode end of the layers and approximately in the middle. Two samples were normally applied side by side, separated by about 2 cm. The lid was placed in position and electrophoresis carried out at 310 v for up to 3 hr. No special cooling precautions were taken and no appreciable drying of the layers occurred during this procedure.

After electrophoresis the layers were air-dried and cut down the middle between the peptide lanes, and ascending chromatography was carried out in the second dimension in freshly prepared butan-1-ol–acetic acid–water (5:2:2, by vol.). The layers were finally air-dried and stained with the cadmium–ninhydrin reagent of Heilman, Barollier & Watzke (1957).

Amino acid analysis. Protein samples were hydrolysed in 5·7 N-HCl under vacuum at 110° for periods of 22 and 72 hr. Amino acid analyses were carried out on the hydrolysates as detailed by Moore & Stein (1963) with a Technicon amino acid analyser. Destruction of serine and threonine was allowed for by extrapolating values obtained at 72 hr. to zero time.

Isolation of total protein. Total protein was isolated after precipitation with 5% trichloroacetic acid by conventional methods detailed by Campbell, Greengard & Kernot (1960).

Determination of total protein. Total protein was determined colorimetrically by the method of Lowry, Rosebrough, Farr & Randall (1951), with crystallized bovine serum albumin as standard.

Measurement of radioactivity. (1) ^{3}H. Protein labelled with ^{3}H was assayed after solution in 0·1 N-NaOH in the silica-gel medium described by Sargent & Campbell (1965). A Packard Tri-Carb model 3003 scintillation spectrometer was used with window settings of 50–1000 and a gain of 60%. In all cases the efficiency of counting was determined after adding a known quantity of [^{3}H]hexadecane to samples that had already been counted. The average efficiency of counting ^{3}H in the present work was 12%.

(2) ^{14}C. Protein labelled with ^{14}C was plated at infinite thinness from formic acid solutions on aluminium disks 1 in. in diameter. Radioassays were performed with a low-background gas-flow Nuclear–Chicago counter (model C115) with an efficiency of approx. 20%. The background recorded with empty planchet holders was 2·3 counts/min. In most cases at least 2000 counts were collected to ensure good statistical accuracy (cf. Loftfield, 1963).

Enzymes. Trypsin and chymotrypsin used to release cytochrome b_5 from microsomes were purchased as crystallized enzymes from Armour Pharmaceutical Co. Ltd. (Eastbourne, Sussex). Trypsin used for 'fingerprinting' studies on cytochrome b_5 was purchased as a three-times-crystallized enzyme from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.). Pronase (*Streptomyces griseus* protease), grade B, was purchased from California Corp. for Biochemical Research (Los Angeles, Calif., U.S.A.). Crude lipase (pancreatin) from hog pancreas was purchased from Koch–Light Laboratories Ltd. (Colnbrook, Bucks.). A partially purified pancreatic esterase was prepared from crude pancreatic lipase by the method of Gelotte (1964), and assayed by the method of Desnuelle, Constantin & Baldy (1955). Dried venoms of *Agkistrodon piscivorus* and *Trimeresurus flavoviridis*, crystalline pancreatic ribonuclease A and crystalline pyruvate kinase were purchased from Sigma Chemical Co. (St Louis, Mo., U.S.A.).

Chemicals. Tris (specially purified) was purchased from British Drug Houses Ltd. (Poole, Dorset). The pH of all tris buffers was recorded at room temperature. The potassium salts of phosphoenolpyruvate, ATP and GTP were purchased from the Boehringer Corp. Ltd. (London, W. 5). These salts were dissolved in water and the pH was adjusted to 7·5 with N-KOH before use.

Haemin (haematin chloride) was purchased in crystalline form from British Drug Houses Ltd. and recrystallized before use by the method of Shemin (1957). Immediately before use the solid was dissolved in a minimal volume of 0·1 N-NaOH and diluted to give a concentration of 0·02 N-NaOH. The concentration of haematin present was determined by the pyridine haemochromogen method as described by Falk (1964). A 0·1 ml. portion of the final haematin solution could be added to 1·0 ml. of a typical microsomal incorporation medium without detectable change in the pH of the latter.

All other chemicals used were AnalaR reagents purchased from British Drug Houses Ltd.

RESULTS

Release of cytochromes from microsomes. As a preliminary to the purification of cytochrome b_5 various methods of releasing the cytochrome from

Table 1. *Effects of various treatments in releasing cytochromes and total protein from rat liver microsomes*

Determinations of total protein, cytochrome b_5 and cytochrome *P*-420 were performed on the supernatants obtained at $105000g_{av.}$ for 120min. after treatment of microsomal suspensions as described in the Materials and Methods section. The concentration of cytochrome b_5 in microsomes was determined to be 0·84mμmole/mg. of microsomal protein, and that of cytochrome *P*-420 was 0·71mμmole/mg. of microsomal protein. These values were based on the release of cytochromes with deoxycholate.

Treatment	Total protein released (%)	Cytochrome b_5 released (%)	Cytochrome *P*-420 released (%)
Trypsin	45·2	76·7	19·4
Chymotrypsin	52·5	64·4	10·5
Pronase	42·2	56·9	10·8
Ribonuclease A	52·3	0·0	0·0
Crude lipase (pancreatin)	53·3	91·8	58·7
Esterase	43·3	0·0	0·0
Venom, *A. piscivorus*	63·6	99·3	89·5
Venom, *A. piscivorus* (heated)	54·3	94·7	79·0
Venom, *T. flavoviridis*	75·5	91·9	92·7
Venom, *T. flavoviridis* (heated)	61·5	84·0	88·8
Sodium deoxycholate	57·6	100	100
Sodium dodecyl sulphate	56·0	84·7	81·5
Ultrasound, freezing-and-thawing	13·0	0·0	0·0

microsomes were investigated. At the same time an opportunity was afforded to study the release of the carbon monoxide-binding pigment, cytochrome *P*-450 (Klingenberg, 1958; Omura & Sato, 1964*a*,*b*). The latter pigment is known to be released from liver microsomes by treatment with snake venoms in the presence of deoxycholate and, during its release, is converted into a form, cytochrome *P*-420, with spectral properties different from those of cytochrome *P*-450 (Omura & Sato, 1964*a*,*b*). The results of this investigation are summarized in Table 1. All enzymes were added such that the ratio of enzyme protein to total microsomal protein was 1:100 and conditions of enzymic action were identical. It may be noted that detergents and preparations such as pancreatin and snake venoms, known to show high phospholipase A activity (Long, 1961), are very efficient in releasing both cytochromes. In contrast, proteases are much more efficient in releasing cytochrome b_5 than in releasing cytochrome *P*-420. Cytochrome b_5 is released somewhat more efficiently by phospholipases than by proteases. Heated venoms, which contain negligible protease activity but essentially unchanged phospholipase activity (Imai & Sato, 1960), are as active in releasing both cytochromes as are unheated venoms. It appears that both cytochromes are firmly bound to microsomal membranes, since treatment with ultrasonic vibrations, followed by freezing-and-thawing, does not release either cytochrome. The failure of ribonuclease or a partially purified esterase from pancreas to release either cytochrome demonstrates that the active principles of pancreatin are phospholipase(s) and proteases.

Isolation and properties of cytochrome b_5. Trypsin was used to release cytochrome b_5 from microsomes because of the well-established specificity of this enzyme and its ready availability in a pure state. The isolation procedure, which is detailed in the Materials and Methods section, involves ammonium sulphate fractionation, pH precipitation and anion-exchange chromatography. This procedure differs only slightly from that described by Strittmatter & Ozols (1966*a*). The final chromatography step serves to remove two non-coloured contaminating proteins present in small quantities.

'Total' cytochrome eluted from the DEAE-cellulose column had spectral properties over the visible and ultraviolet range identical with those quoted by Strittmatter & Velick (1956) for rabbit liver cytochrome b_5. The preparation sedimented as a single component in the Spinco model E ultracentrifuge in 0·1M-sodium phosphate buffer, pH 6·8, and likewise migrated as a single component on long columns (2cm. × 150cm.) of Sephadex G-75 in 0·1M-phosphate buffer, pH 6·8. Analysis of the preparation by electrophoresis on starch gel, polyacrylamide gel or cellulose acetate at pH 4·5 or 8·5, however, showed the presence of two components that stained positively for both protein and haem. No other components were detected. When eluted from cellulose acetate and run again under identical conditions each component behaved as a single

Fig. 1. Cellulose acetate electrophoresis of 'total' cytochrome b_5 and its separated components F and S. 'Total' cytochrome b_5, cytochrome b_5F and cytochrome b_5S were isolated on DEAE-cellulose as detailed in the Materials and Methods section. Electrophoresis was carried out in the glycerol–sodium barbiturate buffer, pH 8·6, as detailed in the Materials and Methods section. Zones depicted stained positively for both protein and haem.

species with unchanged mobility (Fig. 1). The two cytochrome components could also be resolved by prolonged chromatography on DEAE-cellulose and were found to have spectral properties identical with those of 'total' cytochrome b_5. The component cytochrome b_5F had a higher mobility than the component cytochrome b_5S on DEAE-cellulose at pH 6·8. Cytochrome b_5S migrated towards the anode at a faster rate than cytochrome b_5F on electrophoresis at pH 8·6 (Fig. 1). These data demonstrate that the two components do not exist in a monomer–dimer relationship, but differ in electrical charge.

Amino acid analysis of cytochrome b_5. Amino acid analyses carried out on 'total' cytochrome b_5 and the two components F and S are summarized in Table 2. Hydrolysis in the presence of haem caused marked destruction of certain amino acid residues, in particular those of tyrosine and histidine, and the analyses quoted here were carried out on the apoproteins after removal of haem. It is apparent that all three species of cytochrome b_5 show markedly similar amino acid compositions, a striking feature being the complete absence of sulphur-containing amino acids. It appears from the results that cytochrome b_5F contains one fewer

Table 2. *Amino acid analyses of 'total' cytochrome* b_5 *and its components F and S*

Amino acid analyses were performed on the apoproteins as described in the Materials and Methods section. Results for 'total' cytochrome b_5 are the means of two separate analyses, and results for cytochrome b_5F and cytochrome b_5S are the means of four separate analyses. Results are corrected for destruction of serine and threonine. Numbers of residues/mol. of protein were calculated to the nearest integer. —, Not determined.

	'Total' cytochrome b_5		Cytochrome b_5F		Cytochrome b_5S	
	(moles/100 moles)	(residues/mol.)	(moles/100 moles)	(residues/mol.)	(moles/100 moles)	(residues/mol.)
Asp	11·0	8	10·9	8	11·9	9
Thr	6·3	5	6·8	5	6·6	5
Ser	6·2	5	7·3	6	6·4	5
Glu	16·2	12	16·3	12	17·8	13
Pro	3·5	3	3·7	3	3·8	3
Gly	8·1	6	7·7	6	8·0	6
Ala	4·9	4	4·9	4	4·9	4
Val	5·4	4	5·4	4	5·4	4
CyS	0·0	0	0·0	0	0·0	0
Met	0·0	0	0·0	0	0·0	0
Ile	3·8	3	3·4	3	3·7	3
Leu	8·4	6	7·7	6	8·0	6
Tyr	4·2	3	3·9	3	3·5	3
Phe	2·6	2	2·3	2	2·4	2
Lys	9·0	7	9·3	7	8·2	6
His	7·5	6	7·2	5	6·7	5
Arg	4·4	3	4·2	3	4·1	3
Trp	—	(1)*	—	(1)*	—	(1)*
Total		78		78		78

* 1 residue of tryptophan/mol. of calf liver cytochrome b_5 is quoted by Strittmatter & Ozols (1966*a*).

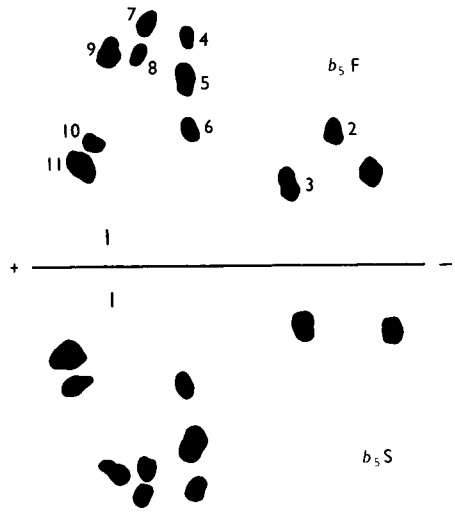

Fig. 2. Tryptic 'fingerprint' patterns of cytochrome b_5F and cytochrome b_5S. The cytochrome b_5 components were separated on DEAE-cellulose, the apoproteins digested with trypsin and the resulting peptides separated on thin layers as detailed in the Materials and Methods section. Peptide zones shown stained positively with ninhydrin.

aspartic acid, one more serine, one fewer glutamic acid and one more lysine residue than cytochrome b_5S.

Effects of trypsin on cytochrome b_5 *and apocytochrome* b_5. Exposure of native cytochrome b_5 to trypsin with a 1:50 ratio of enzyme to protein caused no detectable hydrolysis. This was true whether the reaction was carried out in 0·5M-ammonium hydrogen carbonate or in the autotitrator at pH 9·0. No peptides were detected after such treatment by 'fingerprinting' studies. Heating cytochrome b_5 at 100° for 7min. at pH 6·8 did not increase the susceptibility of the protein to trypsin.

In contrast with the above findings apocytochrome b_5 was markedly susceptible to trypsin. Analyses of tryptic digests of cytochrome b_5S and cytochrome b_5F, carried out on thin layers of silica gel G under identical conditions, are shown in Fig. 2. It is apparent that tryptic digestion of apocytochrome b_5S yields ten ninhydrin-positive peptides and that an additional peptide appears in digests of apocytochrome b_5F. The number of peptides obtained from the cytochromes agree well with the presence of 6 lysine residues and 3 arginine residues in cytochrome b_5S and an additional lysine residue in cytochrome b_5F.

Biosynthesis of cytochrome b_5 in vivo. Despite the microheterogeneity apparent in cytochrome b_5 isolated under the present conditions, it was considered that the protein was sufficiently homogeneous to warrant investigation of its biosynthesis. The purification procedure used, moreover, was applicable to amounts of liver of about 10g. and so could be applied on a routine basis.

L-[4,5-^{3}H]Leucine was injected intravenously into groups of three rats and cytochrome b_5 isolated from both the rough and smooth fractions of microsomes from the pooled livers. Over many isolation procedures it was apparent that approximately equal amounts of the cytochrome could be isolated directly from both rough and smooth microsome fractions. On average 0·15mμmole of cytochrome b_5 was isolated/mg. of microsomal protein, whether from total microsomes or from rough and smooth microsomal fractions. In all cases the two subfractions F and S of cytochrome b_5 were observed in both rough and smooth fractions, but these subfractions were not isolated separately. Approximately equal amounts of the two subfractions were observed over the entire study.

Variations were found in the absolute specific radioactivities of both cytochrome b_5 and total protein in individual experiments, such variations being especially marked at short times. For this reason the results in Fig. 3 are expressed as the ratio of specific radioactivities in rough and smooth fractions, measured in individual experiments. In general the specific radioactivity of total protein did not differ greatly from that of cytochrome b_5. Thus the mean specific radioactivity of total microsomal cytochrome b_5 in all experiments was 32000 disintegrations/min./mg., whereas the mean specific radioactivity of total protein was 16000 disintegrations/min./mg.

It is apparent that at short times after injection the specific radioactivity of cytochrome b_5 in the rough fraction exceeds that of cytochrome b_5 in the smooth fraction, the ratio of the specific radioactivities being maximal at about 5min. By approx. 14min. the specific radioactivities of cytochrome b_5 in the two fractions are equal and beyond this time the specific radioactivity is somewhat greater in smooth than in rough microsomes. The specific radioactivities are essentially equal once more after 24hr. It may be noted that the kinetics of labelling of total protein are qualitatively very similar to those for cytochrome b_5.

Such results are consistent with a precursor–product relationship existing between cytochrome b_5 in the rough and smooth fractions. We conclude that cytochrome b_5 is synthesized initially in the rough fraction of microsomes and only subsequently appears in the smooth fraction.

Incorporation of amino acids into cytochrome b_5 *in isolated microsomes.* Though the foregoing results demonstrate, as expected, that cytochrome b_5 originates in the rough-surfaced fraction of microsomes, they give no insight as to how the

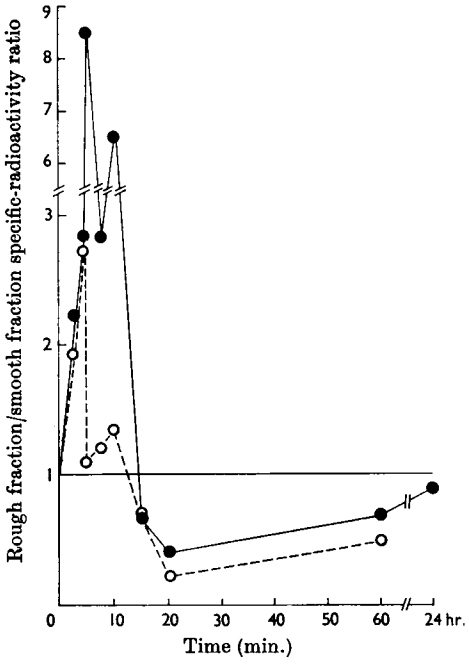

Fig. 3. Specific radioactivities of total protein and cytochrome b_5 in rough and smooth fractions of microsomes after administration of a single dose of [^{3}H]leucine to rats. Groups of three rats, each of which had received an intravenous injection of 300μc of L-[^{3}H]leucine (35100mc/mmole), were killed at times shown, and the ratios of specific radioactivities of cytochrome b_5 in rough and smooth fractions of microsomes determined. The ratios for total protein in rough and smooth fractions were determined in the same experiments. Experimental details are described in the Materials and Methods section. ○, Total protein; ●, cytochrome b_5.

protein is integrated into microsomal membranes and, more important, no insight as to how the protein appears in smooth-surfaced microsomes. In an attempt to shed light on these problems our efforts were next directed to testing whether isolated microsomes were capable of synthesizing cytochrome b_5. A conventional microsomal system, as detailed in the Materials and Methods section, was used for amino acid-incorporation studies except that the cell sap was passed through Sephadex to decrease the endogenous amino acid pool, and so increase the initial specific radioactivity of the added labelled amino acid in the system.

Table 3 demonstrates that, when microsomes are incubated under standard conditions, recovered from the medium and washed and cytochrome b_5 is isolated in the usual way, significant radioactivity is detected in the cytochrome. Both lysine and leucine serve to label cytochrome b_5. The specific radioactivity in the cytochrome is about one-fifth of that in total protein. It is very unlikely that these essential amino acids will serve as precursors for haem biosynthesis in the present experiments, and Table 3 shows that negligible radioactivity is present in haem.

In two cases in Table 3 subfractions S and F were separated on DEAE-cellulose and specific radioactivities in the separated fractions determined. The differences in specific activities were considered not to be significant and for this reason the remainder of the work was concerned solely with 'total' cytochrome b_5.

We are concerned here with biosynthesis of the protein moiety of the cytochrome, but it is the intact cytochrome that is being isolated. The results therefore assume that haem can be easily added on to the newly synthesized apoprotein under the present conditions. Table 4 demonstrates that haem added to the system to a final concentration of 0·1mM, i.e. sufficient to couple with about 1mg. of newly synthesized apoprotein, has negligible effect on the incorporation of amino acid into total protein, and only a small stimulatory effect on amino acid incorporation into cytochrome b_5. Such a result implies first that there is sufficient haem present in the system to couple with most of the newly synthesized apoprotein, and further that coupling of haem and apoprotein occurs readily under the present experimental conditions. Strittmatter (1960) has shown that formation of cytochrome occurs readily at neutral pH values after mixing solutions of apocytochrome b_5 and haem.

'Fingerprinting' of radioactive cytochrome b_5. Though the foregoing results are entirely consistent with biosynthesis of cytochrome b_5 occurring in isolated microsomes, it is highly desirable to demonstrate that the radioactive amino acid has been incorporated into peptide linkage in the protein. This is particularly true in the present experiments where, in addition to the hazards of the purified cytochrome being contaminated with traces of radioactive amino acid, there is the further hazard of contamination with small radioactive tryptic peptides formed during extraction of the cytochrome from microsomes with trypsin.

Cytochrome b_5 was isolated in the usual way from microsomes that had been incubated in the presence of [^{14}C]lysine. The isolated cytochrome had a specific radioactivity of 394 counts/min./mg. Haem was removed from the cytochrome, and the peptides formed after tryptic digestions were separated on thin layers of silica gel G as described in the Materials and Methods section. After staining with ninhydrin the usual pattern was apparent (see Fig. 2). Since 'total' cytochrome b_5 was

Table 3. *Incorporation of amino acids into total protein and cytochrome* b_5 *in isolated microsomes*

The incorporation medium described in the Materials and Methods section (total volume 1·0 ml.) was scaled up 30-fold. The following amounts of labelled amino acids were used: in Expt. *A* 35 μc of L-[^{14}C]lysine (180 mc/m-mole); in Expt. *B* 30 μc of L-[^{14}C]lysine (150 mc/m-mole); in Expt. *C* 60 μc of L-[^{14}C]lysine (324 mc/m-mole); in Expt. *D* 30 μc of L-[^{14}C]lysine (180 mc/m-mole); in Expt. *E* 60 μc of L-[^{14}C]leucine (155 mc/m-mole). The reaction was terminated by adding the unlabelled counterpart of the radioactive amino acid to a concentration of 1 mM and rapidly chilling the suspension. Microsomes were sedimented and washed, and cytochrome b_5 was isolated without addition of carrier protein, as described in the Materials and Methods section.

Expt.	Precursor amino acid	Total protein (counts/min./mg.)	Cytochrome b_5 (counts/min./mg.)	Haem (% of total counts/min. in cytochrome b_5)
A	Lysine	1700	456	—
B	Lysine	1270	391	2
C	Lysine	1680	200	0
D	Lysine	925	50	0
E	Leucine	1770	233	2

Table 4. *Effects of haem on the incorporation of* [^{14}C]*leucine into total protein and cytochrome* b_5 *in isolated microsomes*

The incorporation medium was as described in the Materials and Methods section with microsomes equivalent to 1 g. wet wt. of liver in a total volume of 1·0 ml.; 10 μc of L-[^{14}C]leucine (155 mc/m-mole) was present. The reaction was terminated by chilling and at the same time addition of unlabelled leucine to a concentration of 1 mM, together with unlabelled microsomes equivalent to 5 g. wet wt. of liver. Microsomes were recovered, a sample was removed for isolating total protein and the remainder was digested with trypsin. Cytochrome b_5 was isolated from the tryptic digest in the usual way after adding 400 μg. of unlabelled cytochrome as carrier.

Expt.	Total protein (counts/min./mg.)	Cytochrome b_5 (counts/min./mg.)
Energy source absent	5	4
Energy source present	543	16
Energy source and haem (0·1 mM) present	509	19

isolated, 11 peptides, including peptide 2, were detected. Peptides 7 and 8 were not well resolved in this case and were taken as a single zone. The peptides, together with blank areas, were eluted with 50% (v/v) methanol and counted at infinite thinness on the low-background counter. The results are shown in Table 5, where count rates are significant only if they exceed the blanks by five times the standard deviation of the latter [see *Notes on Low Level Beta Counting* (1961), published by Tracerlab, Waltham, Mass., U.S.A.]. On this basis the origin area and peptides 9, 10 and 1 do not contain significant radioactivity. Therefore seven out of the 11 tryptic peptides of apocytochrome b_5 are labelled in this experiment.

Table 5. *Count rates in peptides isolated from cytochrome* b_5 *labelled with* [^{14}C]*lysine in isolated microsomes*

Microsomes were incubated as described in Table 3 in a total volume of 30 ml. with 30 μc of [^{14}C]lysine (150 mc/m-mole), and cytochrome b_5 (394 counts/min./mg.) was isolated without addition of carrier protein. Tryptic 'fingerprints' were prepared from the apoprotein and ninhydrin-positive peptide areas, together with four separate blank areas, were eluted, plated on aluminium planchets and counted in a low-background counter (Nuclear–Chicago model C115). A total of 900 counts was collected from each sample. The mean count rate of the four blanks, 2·6 $\pm$ 0·2 (S.D.) counts/min., was subtracted from the mean of each sample to give the mean count rates shown. Peptide 0 represents the ninhydrin-negative origin of the 'fingerprint'; otherwise peptides are numbered as in Fig. 2.

Peptide no.	Mean counts/min. $\pm$ S.D.
5	5·4 $\pm$ 0·34
11	3·2 $\pm$ 0·28
2	3·2 $\pm$ 0·28
4	3·0 $\pm$ 0·27
7/8	2·1 $\pm$ 0·25
3	1·6 $\pm$ 0·24
6	1·4 $\pm$ 0·24
1	1·0 $\pm$ 0·22
10	0·5 $\pm$ 0·22
9	0·2 $\pm$ 0·22
0	0·2 $\pm$ 0·22

It is noteworthy that about 20 counts/min. was recovered from the thin layer. On the basis of the staining intensity with ninhydrin it was estimated that approx. 100 μg. of protein was applied to the layer in the first instance. On this basis the specific radioactivity of the protein would be 200 counts/min./mg., a value that agrees comfortably with the

specific radioactivity determined for the intact cytochrome, since losses are inevitable during handling of the small quantities of material available in experiments of this type.

We conclude from this experiment that [^{14}C]-lysine is incorporated into peptide linkage in cytochrome b_5 in an isolated microsomal amino acid-incorporating system.

DISCUSSION

The first aspect of the present work that requires discussion concerns the nature of the two components of cytochrome b_5 isolated from liver microsomes. Agents that have been used in the past to release cytochrome b_5 from microsomes include detergent (Strittmatter & Ball, 1952), pancreatin and purified phospholipase A (Strittmatter & Velick, 1956; Strittmatter, 1960) and trypsin (Phillips & Langdon, 1962; Poltoratsky-Bois & Chaix, 1964). The present results (Table 1) confirm these findings and show that phospholipase A is somewhat more effective in releasing the cytochrome than is trypsin under the conditions used. Though it had been accepted that cytochrome b_5 isolated from calf liver microsomes was homogeneous by several physical, enzymic and spectral characteristics (Strittmatter, 1963), Strittmatter & Ozols (1966*a*) have since shown that the purified haemoprotein contains two remarkably similar components. These two components were obtained from calf liver microsomes after treatment with phospholipase A whether in the presence or absence of trypsin inhibitor. The two components differed in that one had an additional glutamylserine sequence at its *C*-terminus. Treatment of the two components with trypsin caused the cleavage of peptide bonds at both the *N*- and *C*-termini to yield a large single limit peptide containing haem; this peptide had the same spectral properties as the original components. The present results demonstrate that two very similar components of cytochrome b_5 can be isolated after treatment of rat liver microsomes with trypsin, but that further treatment with trypsin at 37° for up to 4hr. did not cause detectable cleavage of peptide bonds.

Though it is difficult to place complete reliance on the amino acid analyses carried out on the limited amounts of total protein available in the present work, it would appear that significant differences occur in the serine, glutamic acid, aspartic acid and lysine contents of the two components (Table 2). A difference in the lysine contents is definitely indicated by the 'fingerprinting' studies (Fig. 2), which show the presence of an extra basic peptide in component F, and the finding that this peptide is radioactive (Table 5) after labelling of microsomes with [^{14}C]lysine.

Though the present results therefore do not define the precise differences between the two components, they demonstrate their marked similarity and further show that the two components can be isolated from both the rough and smooth fractions of rat liver microsomes. Both the results quoted here and those of Strittmatter & Ozols (1966*a*) are consistent with the interpretation that tryptic action is not the direct cause of the appearance of the two components. In this event it is pertinent to ask whether the heterogeneity of cytochrome b_5 is caused by the action of an unidentified microsomal protease, or whether the protein actually exists in two different forms in microsomal membranes *in vivo*. Further work is required to resolve this problem.

The second aspect of the work that requires discussion concerns the biosynthesis of cytochrome b_5 *in vivo*. It has already been shown that certain constitutive proteins of the membranes of the endoplasmic reticulum of liver are synthesized initially in the rough fraction and only subsequently transferred to the smooth fraction. Thus the appearance of glucose 6-phosphatase and NADH–cytochrome *c* oxidoreductase activities has been investigated in developing rat hepatocytes (Dallner *et al.* 1966*a*,*b*), and the induction of NADPH–cytochrome *c* oxidoreductase and the carbon monoxide-binding pigment has been studied in liver after repeated administration of phenobarbitone to rats (Orrenius, 1965; Ernster & Orrenius 1965). Whereas these studies used enzymic and spectrophotometric assays to detect the appearance of newly synthesized protein, the present experiments are based on the direct isolation of cytochrome b_5 after administration of a single dose of radioactive amino acid to rats. The results obtained by the latter method are in accord with the previous conclusion that constitutive proteins of the endoplasmic reticulum appear initially in the rough fraction. Omura, Siekevitz & Palade (1967) have quoted their own unpublished results showing that both cytochrome b_5 and NADPH–cytochrome *c* oxidoreductase originate in the rough endoplasmic reticulum. These results also were based on direct isolation of the proteins after administration of a single dose of radioactive amino acid to rats.

It is apparent from the present results (Fig. 3) that newly synthesized cytochrome appears in microsomes within minutes after administration of [^{3}H]leucine to animals. This is consistent with a rapid rate of synthesis of the protein, which at first sight is at variance with the relatively long half-life of 120hr. quoted for cytochrome b_5 in rat liver (Omura *et al.* 1967). It therefore appears that, though the bulk of cytochrome b_5 remains unaltered for relatively long periods, there is at any given time a rapid and necessarily small amount of

synthesis of new cytochrome. Fig. 3 also demonstrates that the specific radioactivities of cytochrome b_5 in rough and smooth microsomes are approaching equality after about 1 hr. This reflects a rapid transfer of newly synthesized cytochrome from the rough to the smooth membranes, and is consistent with the formation of smooth membranes from rough membranes by rapid removal of ribosomes attached to the rough membranes (Orrenius, 1965; Omura *et al.* 1967). It must be admitted, however, that such results are also consistent with a rapid transfer (e.g. by diffusion) of new synthesized cytochrome b_5 from polysomes in the rough fraction to rough membranes, and a slightly less rapid transfer from the same polysomes to the smooth membranes.

The final aspect of the discussion concerns the cell-free biosynthesis of cytochrome b_5. From the results presented in Tables 3 and 4 it may be stated that isolated microsomes will catalyse the energy-dependent incorporation of amino acids into cytochrome b_5. It is known, moreover, that unlabelled cytochrome b_5 can be added to the incorporation medium, either at the start or at the end of the incubation, and that cytochrome b_5 subsequently recovered from the soluble phase of the medium is not radioactive. Such addition of unlabelled cytochrome does not dilute the specific radioactivity of cytochrome b_5 isolated from the microsomes in the usual way (J. R. Sargent & B. P. Vadlamudi, unpublished work). We conclude therefore that the incorporation of radioactive amino acids into cytochrome b_5 observed in Tables 3 and 4 is meaningful, and that isolated microsomes will synthesize the cytochrome to at least a limited extent.

The above interpretation is reinforced by the demonstration that [^{14}C]lysine is incorporated into peptide linkage in the cytochrome (Table 5). It is noteworthy that on average the specific radioactivity of cytochrome b_5 is about one-fifth of that of total protein in the isolated microsomal system (Table 3). Moreover, the average yield of cytochrome b_5 isolated from microsomes without adding carrier cytochrome was about 25%. For these reasons only limited quantities of cytochrome b_5 of limited specific radioactivity were available for the work described in Table 5, and this is reflected in the low count rates quoted in the Table. It must be emphasized, however, that count rates exceeding 1·0 count/min. are highly significant statistically and that significant radioactivity is associated only with ninhydrin-positive material. The data in Table 5 suggest that all the lysine-containing peptides are radioactive to some extent, which implies that the observed incorporation represents considerable completion of polypeptide chains pre-existing on polysomes. It is not possible from the results, however, to state whether initiation of polypeptide chains has occurred.

It may be noted finally that Campbell (1967) has reported the biosynthesis of NADPH–cytochrome c oxidoreductase in isolated rat liver microsomes. The latter finding, together with the present results, may indicate that at least some steps in the assembly of proteins associated with membranes may be fruitfully investigated at the cell-free level. The present results, however, define neither the site of binding of the newly synthesized cytochrome to microsomes, nor the manner in which it is bound. The extent to which the cell-free synthesis of cytochrome b_5 reflects stages in the assembly of the microsomal membrane therefore must await further work.

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