Biosynthesis of Microsomal Nicotinamide-Adenine Dinucleotide Phosphate-Cytochrome *c* Reductase by Membrane-Bound and Free Polysomes from Rat Liver

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1. NADPH-ferricytochrome *c* oxidoreductase (EC 1.6.2.3) was purified from the endoplasmic reticulum of rat liver cells. The methods, which involved digestion of membrane with Steapsin, a crude pancreatic extract containing diastase and trypsin, gel filtration and preparative electrophoresis on polyacrylamide, provided an enzyme with a high specific activity in good yield. 2. The incorporation of ¹⁴C-labelled amino acids into the purified reductase by the incubation of various subcellular fractions was studied. The microsome fraction, bound polysomes, free polysomes and detergent-treated polysomes effected the synthesis of the enzyme. 3. The reductase that had been synthesized by the polysomes was tightly bound to preparations of smooth-surfaced endoplasmic reticulum that were added to the incubation medium. 4. Reductase activity could be detected on both free and detergent-treated polysomes. Evidence is presented to show that this activity was due, at least in part, to the presence on the ribosomes of nascent enzyme. The association of enzyme with detergent-treated polysomes did not appear to be due to contamination of the ribosomes with either membrane or cell sap but it is possible for such ribosomes to adsorb some enzyme. 5. The amount of reductase activity associated with the detergent-treated polysomes was increased when the rats from which the polysomes were derived had been previously injected with phenobarbitone. 6. The results are discussed with respect to their relevance for the question of the existence of two functionally different groups of polysomes in the liver and for current ideas on the biogenesis of membranes.

Recent studies on the biogenesis of membranes in the liver cell have suggested that constitutive membrane proteins are synthesized by the rough-surfaced endoplasmic reticulum (presumably by membrane-bound ribosomes) and only subsequently appear in the smooth endoplasmic reticulum, perhaps because of a transition from the rough to the smooth type of membrane by detachment of ribosomes (Dallner, Siekevitz & Palade, 1966*a,b*). There has been increasing evidence that the liver cell contains polysomes that are not associated with the rough-surfaced endoplasmic reticulum, the so-called free polysomes. At present it is not clear whether the two types of polysomes reflect differences in function or perhaps indicate some kind of control mechanism (Loeb, Howell & Tomkins, 1967).

In an attempt to test some of the hypotheses concerning membrane biogenesis and at the same time to elaborate the relationship between

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membrane-bound and free polysomes we have studied the biosynthesis *in vitro* of the constitutive membrane protein, NADPH-ferricytochrome *c* oxidoreductase (EC 1.6.2.3), by various subcellular preparations. We report the incorporation of ¹⁴C-labelled amino acids into this enzyme by membrane-bound, free and detergent-treated polysomes.

Preliminary reports of some aspects of this work have already appeared (Campbell, Lawford & González-Cadavid, 1967*a*; Campbell & Lawford, 1968).

MATERIALS AND METHODS

Chemicals. Chemicals were obtained from British Drug Houses Ltd., Poole, Dorset, and were of AnalaR grade wherever possible. Horse-heart cytochrome *c* (grade 111) and Steapsin (a crude pancreatic extract containing diastase and trypsin) were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. The scintillators were purchased from Nuclear Enterprises Ltd., Sighthill, Edinburgh. Puromycin dihydrochloride was purchased from Nutritional

Biochemicals Corp., Cleveland, Ohio, U.S.A. Reduced NADP was purchased from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Other chemicals were as specified by Campbell, Lowe & Serck-Hanssen (1967b).

Radiochemicals. The following was purchased from The Radiochemical Centre, Amersham, Bucks.: U-¹⁴C-labelled amino acid mixture containing alanine, arginine, aspartic acid, glutamic acid, glycine, leucine, isoleucine, lysine, phenylalanine, proline, serine, threonine, tyrosine and valine with a specific activity of 54mc/mg.-atom of C.

Animals. The rats used in these studies were male, Wistar strain, and were purchased from Tuck and Sons Ltd., Rayleigh, Essex. The rats weighed between 200 and 300 g. and were starved overnight before being killed. Animals were given an intraperitoneal injection of phenobarbitone (80 mg./kg. body wt.) at 48 and 24 hr. before being killed. In these experiments both control and phenobarbitone-treated rats were starved for 48 hr. Administration of ¹⁴C-labelled amino acid was also by interperitoneal injection 4 min. before the animals were killed.

Subcellular fractionation. The rats were stunned by a blow to the head, decapitated and their livers quickly transferred to ice-cold homogenizing medium. The livers were blotted with filter paper and passed through a tissue press (purchased from Climpex Ltd., London, N.W. 7; made to the design of Porterfield, 1960). The liver mince was homogenized in 2 vol. of medium for the preparation of microsomes, cell sap or smooth endoplasmic reticulum and in 2.5 vol. of medium for the preparation of ribosomes. The homogenizing medium for the preparation of smooth endoplasmic reticulum was 0.25 M-sucrose. In all other preparations the homogenizing medium was 0.25 M-sucrose in medium A. Medium A contained tris-HCl, pH 7.8 (50 mM), KCl (25 mM) and MgSO₄ (5 mM). The tissue suspension was centrifuged at 12000g for 10 min. The supernatant was used for the preparation of the various subcellular fractions as described in (a)–(f).

(a) Microsomes were prepared by centrifuging the 12000g supernatant at 145000g_{av} for 2 hr. After removal of the supernatant (see below) the microsome pellet was drained and the wall of the tube wiped clean with tissue.

(b) Cell sap was obtained by careful aspiration of the microsomal supernatant so as to reject the part near the pellet and the fatty layer at the top. The cell sap was treated to remove amino acids by passing it through a column of Sephadex G-25 equilibrated with sucrose (0.15 M), tris-HCl, pH 7.8 (35 mM), KCl (25 mM) and MgSO₄ (10 mM) (medium B).

(c) pH 5 fraction was prepared from the cell sap (5 ml.) by adjusting the pH to 5.2 with M-acetic acid. The resulting precipitate was collected by centrifugation at 3000g for 5 min. and suspended in 0.5 ml. of tris-HCl, pH 7.8 (0.5 M), followed by the addition of 2 ml. of medium B.

(d) Smooth endoplasmic reticulum was prepared by a modification of the CsCl method of Dallner (1963). The 12000g supernatant was adjusted to 15 mM-CsCl and layered 6 ml. at a time over 6 ml. of 1.3 M-sucrose in 15 mM-CsCl. After centrifugation for 3.5 hr. at 145000g, the smooth endoplasmic reticulum was removed from the interface, diluted 1:1 with cold water and sedimented into a pellet by centrifugation for 60 min. at 145000g.

(e) Membrane-bound and free polysomes were separated by a modification of the method of Webb, Blobel & Potter

(1964). Samples (5 ml.) of the 12000g supernatant were layered over 3 ml. of 0.5 M-sucrose and 4 ml. of 1.6 M-sucrose, both in medium A. When only the free polysomes were required and a comparison with membrane-bound polysomes was not involved, 2.0 M-sucrose in medium A was used in place of the 1.6 M-sucrose. Centrifugation for 3.5 hr. at 145000g yielded the membrane-bound polysomes at the interface and a pellet of free polysomes. The interface layer was collected, diluted with cold medium A and sedimented to a pellet by centrifugation at 145000g for 60 min. The remaining supernatant was removed from the free polysome pellets by suction and the wall of the tube carefully wiped clean with tissue.

(f) Detergent-treated polysomes (C-ribosomes) were prepared by a slight modification of the method of Wettstein, Staehelin & Noll (1963). The 12000g supernatant was adjusted to contain 1.3% deoxycholate and 5 ml. samples were layered over 3.0 ml. of 0.5 M-sucrose in medium A and 4.0 ml. of 2.0 M-sucrose in medium A. After centrifugation at 145000g for 3.5 hr., the supernatant was removed by suction, the polysome pellet drained and the wall of the tube wiped clean with tissue.

Amino acid incorporation by subcellular fractions. Samples (5 ml.) of the appropriate subcellular fraction suspended in medium B (and containing 20–30 mg. of RNA) were incubated at 37° for 30 min. with ATP (2 mM), GTP (0.25 mM), phosphoenolpyruvate (10 mM), pyruvate kinase (50 µg./ml.), cell sap (2 ml.) or pH 5 fraction (1 ml.) and ¹⁴C-labelled amino acids (5 µCi) in a total volume of 10 ml. At the end of the incubation, cold medium B was added and a sample was taken for determination of the total amount of radioactivity incorporated into protein. At this time a preparation of smooth endoplasmic reticulum was added to free and deoxycholate-treated polysome incubations to act as a source of carrier enzyme during the purification procedure and possibly to bind any labelled reductase that had been released from the ribosomes into the soluble phase of the incubation medium. In experiments designed to test the release of nascent protein, the ribosomes were centrifuged into a pellet, smooth endoplasmic reticulum was added to both pellet and incubation supernatant and enzyme was purified from both fractions.

Purification of NADPH-cytochrome c reductase. After incubation for amino acid incorporation the particles were sedimented by centrifugation for 60 min. at 145000g. The particles were washed by homogenization in 12 ml. of potassium phosphate buffer, pH 7.8 (30 mM), containing EDTA (disodium salt, 1 mM) and centrifuged again. The pellets were then resuspended in the phosphate buffer (1 ml./g. of original liver), a sample was taken to measure the radioactivity and Steapsin added to a concentration of 0.7 mg./ml.; the tubes were then sealed. The digestion by Steapsin proceeded for 16 hr. at 4°. The digestion mixture was then centrifuged at 145000g for 60 min. Samples of the supernatant (called 'Steapsin supernatant') were assayed for reductase activity and radioactivity while the remainder of the supernatant was freeze-dried. The dry powder, which possessed all of the enzymic activity of the supernatant, was dissolved in 2–3 ml. of tris-HCl (10 mM) and EDTA (disodium salt, 1 mM), pH 7.8, and passed through a column of Sephadex G-100 (1 cm. × 50 cm.) equilibrated with the same buffer at 4°. Fractions (2 ml.) were collected from the effluent and assayed for reductase activity.

The two fractions showing highest enzymic activity were

further purified by a method of polyacrylamide-gel electrophoresis modified from Shuster & Schrier (1967). The bottom of a glass tube (1 cm. $\times$ 15 cm.) was sealed with Parafilm and 2 ml. of acrylamide (7.5%) in 0.375 M-tris-HCl, pH 8.8, containing EDTA (disodium salt, 1 mM) prepared according to Davis (1964), was allowed to gel in the bottom. The glass tube had three indentations in the wall to prevent the gel sliding out of the tube during the subsequent electrophoresis. On top of this gel was formed a 5 ml. linear gradient of 20–60% (w/v) sucrose in 0.375 M-tris-HCl, pH 8.8, containing EDTA (disodium salt, 1 mM). Another 2 ml. of acrylamide was then layered over the gradient and 0.5 ml. of water layered on top of the acrylamide until the gel had polymerized. The water was then removed by suction. After removal of the Parafilm the glass tube containing the gel was mounted vertically between two electrode tanks containing 25 mM-tris-glycine buffer, pH 8.3, with EDTA (disodium salt, 1 mM). The sample containing the reductase was made 10% in sucrose and up to 4 ml. was layered on the top gel. Electrophoresis proceeded at 5 mA/gel in the cold room (4°) until the yellow band, which identified the reductase, had just migrated into the sucrose gradient. The sucrose fraction containing the yellow band was collected by means of a needle inserted between the upper gel and the wall of the tube. Samples were assayed for radioactivity, protein content and enzymic activity.

Assay of NADPH-cytochrome c reductase. Rate measurements were performed by measuring the rate of change of *E* at 550 m μ at 25° in a model 2000 Gilford recording spectrophotometer. The reaction mixture was similar to that of Phillips & Langdon (1962) and contained potassium phosphate buffer, pH 7.6 (44 mM), KCl (66 mM), KCN (0.1 mM), NADPH (0.06 mM), cytochrome *c* (0.05 mM) and sample in a total volume of 3.3 ml. Control cuvettes contained all the components except NADPH. Enzymic activity was estimated from differences between the initial rates of cytochrome *c* reduction with the complete reaction mixture and with the control under conditions in which the activity was proportional to the enzyme concentration. The reduction of cytochrome *c* was calculated from the molar extinction indices at 550 m μ determined by Margoliash & Frohwirt (1959) of 27.7×10^6 cm.² mole⁻¹ for reduced cytochrome *c* and 9.0×10^6 cm.² mole⁻¹ for the oxidized cytochrome *c*.

One unit of activity is defined as the amount of enzyme that will catalyse the reduction of cytochrome *c* at an initial rate of 1 μ mole/min. in the above system.

Assay of lactate dehydrogenase. Lactate dehydrogenase was assayed by the method of Bergmeyer, Bernt & Hess (1963).

Density-gradient analysis of polysome preparations. Polysomes (9 mg.) were suspended in medium A (1 ml.) and layered on a 29 ml. gradient, 15–50% (w/v) sucrose in medium A. Centrifugation was at 25000 rev./min. for 3 hr. in a SW 25.1 rotor of a Beckman ultracentrifuge. The extinction at 260 m μ was measured in a Gilford model 2000 recording spectrophotometer by pumping the gradient through a flow cell with a constant flow rate of 1 ml./min. Fractions were collected and the enzyme activity was determined.

Measurement of radioactivity. When applicable, samples for determination of radioactivity were adjusted to M-NaOH and left at room temperature for 5 min. to hydrolyse the

RNA before precipitating the protein with a final concentration of 5% (w/v) trichloroacetic acid.

Precipitates were collected by filtration through glass-fibre filters (Whatman GFA), rinsed twice with 3 ml. of 5% (w/v) trichloroacetic acid and twice with 3 ml. of a mixture of chloroform-ether-methanol (2:1:1, by vol.). The filter with the protein was dried at 60° for 15 min. before being placed in a vial containing 10 ml. of scintillation fluid 2,5-diphenyloxazole (4 g./l. of toluene) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (100 mg./l. of toluene). Radioactivity was measured at an efficiency of 66% (background 12 counts/min.) in a Beckman model LS 200B liquid-scintillation spectrometer to a standard error of 5% or better.

The total radioactivity in pure enzyme in the Steapsin supernatant was calculated by multiplying the total counts/min. in enzyme recovered from the gel by the ratio of total enzymic activity in Steapsin supernatant/total enzymic activity recovered from the gel.

Measurement of RNA. The RNA content of the sub-cellular fractions for incubation was determined by the orcinol method of Mejbaum (1939), with hydrolysed yeast RNA as standard.

Measurement of protein. Protein content was measured by the method of Lowry, Rosebrough, Farr & Randall (1951). With the reductase purified by gel electrophoresis, it was necessary to wash the protein free from the high concentration of tris-HCl buffer, which interferes with the Lowry reaction (Shuster & Schrier, 1967). The protein was precipitated in 5% trichloroacetic acid by freezing-and-thawing twice and then centrifuged for 2 hr. at 3000 g. The pellet was washed again in 5% trichloroacetic acid before being dissolved in M-NaOH. Bovine serum albumin standards subjected to similar treatment gave reproducible, linear, standard curves in the range 5–150 μ g.

RESULTS

Isolation of the enzyme. The present procedure, which combines the methods of Jick & Shuster (1966), Omura, Siekevitz & Palade (1967) and Shuster & Schrier (1967), results in a higher average specific activity for the enzyme (Table 1) than has been reported previously.

Table 1. *Purification of microsomal NADPH-cytochrome c reductase*

Specific activity of the enzyme is expressed as μ moles of cytochrome *c* reduced/min./mg. of protein. Results are the average of 20 separate experiments. Recovery per enzyme in the Steapsin digest is taken arbitrarily as 100%.

Stage of purification	Specific activity (units/mg. of protein)	Recovery (%)
Homogenate	0.020	—
Microsome fraction	0.033	—
Steapsin supernatant	1	100
After gel filtration on Sephadex G-100	5–10	95–100
After polyacrylamide gel	29	70

The step involving Sephadex G-100, besides desalting, removed from the Steapsin supernatant a red component (presumably cytochrome *b*₅; see Omura *et al.* 1967) that was the only protein with a rate of migration in the gel similar to that of the reductase. Since the reductase has a yellow colour it could be observed that it was quickly concentrated at the top of the gel when current was applied. Appropriate fractions from the Sephadex column containing 3–5 units of enzyme in volumes up to 4 ml. could be quantitatively fractionated on the gel. The reductase moved as a distinct yellow band, at a rate shown to be much higher than that of the other proteins in the mixture. It was easy therefore to stop the electrophoresis and collect the pure enzyme as soon as it entered the sucrose gradient.

¹⁴C-labelled amino acid incorporation into protein and purified enzyme in vitro by various subcellular fractions. Table 2 presents the average results from three experiments in which the ability of various ribosomal preparations to incorporate ¹⁴C-labelled amino acids *in vitro* were compared. Control experiments demonstrated that the incorporation measured was energy-dependent and proportional to the amount of ribosomal RNA incubated. To isolate the pure reductase it is necessary to have present membranes containing the reductase to act as carrier enzyme and to adsorb any radioactive enzyme present in the incubation medium. For this reason whenever 'free polysomes' or 'deoxycholate-treated polysomes' were used smooth-membrane fraction was added to the incubation mixture at the conclusion of incubation. The total radioactivity recovered in the pure enzyme was not influenced by the amount of 'carrier' membrane added. The total enzyme activity was measured in the Steapsin supernatant, as was that of the enzyme recovered from the gel electrophoresis. For the present purposes it has been assumed that the recovery of enzyme up to the stage of the Steapsin supernatant was uniform in the different experiments and for this reason the recovery to this stage is taken arbitrarily as 100% in Table 1. (It is not meaningful to calculate the recovery of enzyme from the homogenate stage because of the change in the physical state of the enzyme on release from the membranes; see Phillips & Langdon, 1962). The percentage recovery of pure enzyme could then be calculated. The final specific radioactivity of the enzyme in terms of counts/min./mg. of ribosomal RNA incubated could then be calculated from the counts recovered in enzyme multiplied by a factor for the loss of enzyme. Recoveries of enzyme were always better than 70%. The results show that membrane-bound polysomes, free polysomes and even detergent-treated polysomes (C-ribosomes) are approximately equally efficient for the incorporation of radioactive amino acid into NADPH-cytochrome

Table 2. ¹⁴C-labelled amino acid incorporation into reductase protein in vitro by various subcellular fractions

Methods for the preparation of the various subcellular particles are given in the Materials and Methods section. The particles were incubated with a mixture of ¹⁴ C-labelled amino acids in the presence of an energy source. Each incubation tube contained 20–30 mg. of ribosomal RNA. The protein at the various stages in the purification of the enzyme was precipitated with trichloroacetic acid and its radioactivity determined. Radioactivity is expressed as counts/min./mg. of RNA incubated.																
Total incubation					Steapsin digest				Steapsin supernatant				Purified reductase (after gel electrophoresis)			
Micro-somes	Bound poly-somes	Free poly-somes	Deoxy-cholate-treated poly-somes	Incubation mixture contained cell sap	Micro-somes	Bound poly-somes	Free poly-somes	Deoxy-cholate-treated poly-somes	Micro-somes	Bound poly-somes	Free poly-somes	Deoxy-cholate-treated poly-somes	Micro-somes	Bound poly-somes	Free poly-somes	Deoxy-cholate-treated poly-somes
(a) Incubation mixture contained cell sap																
6290	6660	11 690	30 860	2410	3050	3290	12 510	155	102	227	720	4.0	3.9	3.5	6.0	
(b) Incubation mixture contained pH 5 fraction																
18 780	18 270	50 200	20 320	6890	4950	10 610	7560	270	135	426	531	5.9	3.7	2.5	3.4	

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c reductase. An exact comparison of the synthesis of the enzyme by these various fractions, however, is unwarranted since the rate of translation of messenger RNA might be affected by such differences as the presence of the membrane in some incubations (Campbell *et al.* 1967b). That these results are also valid for other membrane proteins is suggested by a comparison of the levels of radioactivity associated with the various fractions at the 'Steapsin digest' and especially at the 'Steapsin supernatant' stage. The 'Steapsin digest' results represent radioactive proteins that are bound so firmly to membranes (or ribosomes) that they have not been removed during the washing with phosphate-EDTA buffer. Digestion with Steapsin (or trypsin) releases only a small percentage of the microsomal proteins, among them NADPH-cytochrome *c* reductase and cytochrome *b*₅ (Phillips & Langdon, 1962; Jick & Shuster, 1966; Omura *et al.* 1967). Thus the Steapsin supernatant could be considered as a mixture of constitutive membrane proteins. The results suggest that the observed synthesis of the reductase is not a special case but is typical of at least a group of membrane proteins.

In some experiments pH5 fraction was used in place of cell sap in the incubation mixture (see Table 2). In these experiments it appeared that a smaller percentage of the total radioactivity incorporated was present in the pure enzyme. Since cell sap was known to contain a factor, absent from the pH5 precipitate, that is necessary for the release of completed polypeptides from ribosomes (Khairallah & Pitot, 1967) it was thought that this could perhaps explain the observed differences. To test whether completed reductase molecules were released into the incubation medium and what influence cell sap might have on this process, two experiments were performed in which the distribution of radioactive reductase between the incubation supernatant and pellet was compared after incubation with cell sap or pH5 fraction. The results are presented in Table 3. It is apparent that at all stages of the purification incubation in the presence of cell sap instead of pH5 fraction results in a higher percentage of radioactive protein being released into the incubation supernatant fraction.

The purification of the reductase from the incubation supernatant fraction depends, of course, on the addition of smooth membranes. For the purification of the enzyme these are then washed with phosphate-EDTA buffer. Thus the radioactivity shown for the incubation supernatant at the 'Steapsin digest' and subsequent stages in the purification represents soluble protein that has been bound so firmly by the added smooth membranes that it is not removed by the wash in phosphate-EDTA buffer. The results indicate that,

Table 3. *A comparison of the release of nascent protein from free polysomes into the incubation medium in the presence of cell sap or pH5 fraction*

Free polysomes were prepared as described in the Materials and Methods section and incubated with a mixture of ^{14}C -labelled amino acids in the presence of an energy source. At the end of the incubation, particle-associated radioactivity was separated from the incubation supernatant by centrifugation. Reductase was purified from both pellet and supernatant fraction after addition of smooth endoplasmic reticulum to each. Protein was precipitated by trichloroacetic acid and the radioactivity determined. Radioactivity is expressed as counts/min./mg. of RNA incubated.

Total incubation				Steapsin digest				Steapsin supernatant				Purified reductase (after gel electrophoresis)			
Pellet	Super- natant	Incubation mixture contained	% of total radio- activity in supernatant	Pellet	Super- natant	Incubation mixture contained	% of total radio- activity in supernatant	Pellet	Super- natant	Incubation mixture contained	% of total radio- activity in supernatant	Pellet	Super- natant	Incubation mixture contained	% of total radio- activity in supernatant
(a) Incubation mixture contained cell sap															
16810	5891	22701	26	5612	605	6127	10	229	29	258	11	1.4	0.9	2.3	39
(b) Incubation mixture contained pH5 fraction															
19552	3890	23442	17	5510	280	5790	5	237	15	252	6	2.6	0.6	3.2	19

Free polysomes were prepared as described in the Materials and Methods section and incubated with a mixture of ¹⁴C-labelled amino acids in the presence of an energy source. At the end of the incubation, particle-associated radioactivity was separated from the incubation supernatant by centrifugation. Reductase was purified from both pellet and supernatant fraction after addition of smooth endoplasmic reticulum to each. Protein was precipitated by trichloroacetic acid and the radioactivity determined. Radioactivity is expressed as counts/min./mg. of RNA incubated.

Table 4. *Association of NADPH-cytochrome c reductase with polysomes*

Results are given as specific activity of enzyme expressed as enzyme unit/mg. of ribosomes. Ribosomal concentrations were estimated with an $E_{1\text{cm}}^{1\%}$ assumed to be 134 at 260m μ . The rats were treated with phenobarbitone as described in the Materials and Methods section.

Source	Free polysomes	Deoxycholate-treated polysomes
Normal rat	0.0066 $\pm$ 0.00049	0.0034 $\pm$ 0.00013
Rat after injection of phenobarbitone	0.0089 $\pm$ 0.00097	0.0077 $\pm$ 0.00080

after incubation in the presence of cell sap, membrane proteins including the reductase are released into the incubation supernatant and subsequently become so firmly attached to the added membranes that they are released only by digestion with Steapsin.

Enzyme activity associated with ribosomes. The assay system used for NADPH-cytochrome c reductase was sufficiently sensitive for the detection of enzyme associated with two of the polysome preparations. Table 4 shows the average number of enzyme units/mg. of ribosomes for 'free polysomes' and for 'deoxycholate-treated polysomes'. The ribosomes used for the assays were centrifuged through 2M-sucrose and both preparations had E_{260}/E_{280} ratios of 1.77-1.82.

An investigation was then made into the significance of the association of enzyme with the ribosomes. Contamination of the ribosome preparation with membrane could have accounted for the presence of enzyme in the free polysome preparations. However, since the enzyme was also present after the disruption of membrane by deoxycholate this is unlikely to be the full explanation. Hence consideration was given to the enzyme being present as a result of contamination of the ribosomal pellet by small amounts of soluble factors containing the enzyme. This was checked by submitting the polysome preparations to density gradient centrifugation. The results shown in Fig. 1 indicate that nearly all the enzyme activity was associated with the polysome-containing fractions. In fact, of the total enzyme activity recovered from the gradient, 88% was recovered in the polysome fractions and only 12% was in the lighter fractions. It will be seen that the enzyme was associated with polysomes of all sizes.

The above results with reductase were in marked contrast with those obtained with lactate dehydrogenase (EC 1.1.1.27), chosen as a typical soluble enzyme. When 9mg. of deoxycholate-treated polysomes having a total lactate de-

Fig. 1. Distribution of NADPH-cytochrome c reductase and polysomes in a sucrose density gradient. The conditions for the gradient centrifugation and collection of fractions for enzyme assay are given in the Materials and Methods section.

hydrogenase activity of 1 unit (μ mole of pyruvate reduced/min.) were subjected to centrifugation on a sucrose density gradient, all the lactate dehydrogenase activity was found in the lighter fractions and none was present in the fractions containing the polysomes.

The above results suggest that the association of reductase with ribosomes was not due to contamination of the pellets with soluble factors. To determine the ability of ribosomes to adsorb reductase use was made of the effect of phenobarbitone to enhance the enzyme content of the endoplasmic reticulum (Orrenius, Ericsson & Ernster, 1965). Polysome preparations obtained from rats that had been treated with phenobarbitone had more enzyme activity than normal preparations (see Table 4). The polysomes from the phenobarbitone-treated rats were submitted to density gradient centrifugation. Both the polysome-containing fraction and the lighter fractions had enhanced enzyme activity but the distribution of activity between the two groups of fractions was as with normal polysomes. Normal deoxycholate-treated polysomes were resuspended in the deoxycholate supernatant from the polysome preparation from phenobarbitone-treated rats. The polysomes were then isolated again. The results obtained showed that the activity of normal polysomes (0.0033) could be enhanced to the same activity as that shown by the polysomes from phenobarbitone-treated rats (0.0090). By repeating the centrifugation of such polysomes through 2M-sucrose the activity was reduced to 0.0062. When these experiments were performed with normal polysomes and deoxycholate-treated supernatant from normal rats there was no enhancement of the enzymic activity on the re-isolated polysomes.

The above results indicated that polysomes from normal rats are able to adsorb more enzyme from

Table 5. *Effect of puromycin on the release of cytochrome c reductase from deoxycholate-treated polysomes*

Deoxycholate-treated polysomes were obtained from normal rats and incubated at a concentration of 3mg./ml. under the standard conditions for amino acid incorporation with the omission of ^{14}C -labelled amino acids for 15 min. at 37° and in the presence of 0.7mm-puromycin. Radioactivity is expressed as counts/min./mg. of ribosome. The number of experiments, the results of which were averaged, are shown in parentheses.

	Enzymic activity	Release (%)	Radio-activity	Release (%)
Before incubation	0.0040		546	
After incubation	0.0020 (6)	50	238 (4)	51

disrupted membranes. The effect of pH on the adsorption of enzyme to ribosomes was tested as follows. Deoxycholate-treated polysomes from normal rats were resuspended in the deoxycholate supernatant from the preparation of polysomes from phenobarbitone-treated rats, the pH of the supernatant being adjusted from 6.5 to 9.5 at steps of 0.5 pH unit. The pH of the supernatant produced under normal conditions was 8.0 at 2° and it was between pH 8.0 and 8.5 that the maximum adsorption of enzyme took place. At lower pH values the adsorption was markedly less.

Another means by which reductase might be associated with ribosomes would be as a result of its synthesis, i.e. present as nascent protein. In view of the known action of puromycin on the release of nascent protein from ribosomes (Nathans, 1964) its effect on the release of reductase from the polysome preparations was determined. Radioactive nascent protein was obtained by injection of rats with ^{14}C -labelled amino acids and killing the animals after 4 min. The polysomes containing radioactive nascent protein were isolated from the livers. The ribosomes were incubated for 15 min. at 37°, in the usual amino acid-incorporation system with 0.7mm-puromycin in place of ^{14}C -labelled amino acids, and were then centrifuged down at 200 000g for 2 hr. through 1 ml. of M-sucrose in medium A. The results of a series of experiments are shown in Table 5. It will be seen that the ribosomes lost 50% of their reductase activity and a comparable proportion of radioactive nascent protein.

DISCUSSION

The results of the experiments on the synthesis of NADPH-cytochrome *c* reductase will be discussed in three parts: firstly, the evidence that the results provide for the synthesis of reductase by the isolated polysome preparations; secondly, their bearing on

the functional significance of free and bound polysomes; finally, in relation to current ideas concerning the biogenesis of the membranes of the endoplasmic reticulum.

After the incubation of the various subcellular fractions the reductase was carefully purified and radioactivity was found to remain with the enzyme. (Since relatively large amounts of polysomes were subjected to incubation and a highly efficient method of radioactive counting was utilized the total counts measured in each sample were always in excess of 15 above background.) In view of the high resolving power of electrophoresis on polyacrylamide gel we are satisfied that the reductase was free from any known contaminants. The specific enzyme activity obtained always was higher than that for any preparation previously reported. In spite of the care taken with the purification, the possibility that the radioactivity was due to contamination with an unknown radioactive protein must be considered. For this purpose we may compare the counts found in the total incubation of the various preparations (Table 2) with those actually found in the pure reductase and see whether there is a reasonable correlation with the expected recovery. From Table 1 we see that the pure enzyme is about 30 times more active per milligram of protein than that in the Steapsin supernatant. On this basis about 3% of the total protein in the Steapsin supernatant would be due to enzyme. Thus if the enzyme had the same specific radioactivity as the average protein in the digest one would expect about 3% of the total radioactivity to be found in the purified enzyme. In fact, for incubation in cell sap the percentage found for microsomes was 4.0 out of 155 (2.58%). Comparable figures for the other preparations are 3.82% for bound polysomes, 1.54% for free polysomes and 0.80% for deoxycholate-treated polysomes. For incubation in the presence of pH 5 fraction the figures are 2.19, 2.74, 0.59 and 0.64. Thus, with the exception of deoxycholate-treated polysomes and free polysomes incubated in pH 5 fraction, the amount of radioactive amino acid associated with the purified enzyme was much as would be expected in the event of the enzyme being synthesized at an average rate.

The other results that bear on the synthesis of enzyme by the isolated polysomes are from the experiments in which enzyme was detected on the ribosomes. It is clear that under certain conditions the ribosomes can adsorb enzyme released when the membranes are disrupted by deoxycholate. The nature of the linkage between reductase and the ribosomes is of particular interest. It seems unlikely to be ionic for the electrophoretic mobility of the reductase and its amino acid composition

(G. Ragnotti, G. R. Lawford & K. Brew unpublished work) suggest that the protein is acidic. In view of this the effect of increasing adsorption with increasing pH would be contrary to that expected from an ionic linkage and is more likely to be explained by the effect of the high pH on the structure of the ribosomes. Once having been adsorbed on to the ribosomes the enzyme is not released under the conditions used in our experiments.

Although the above results suggest that some of the association of the enzyme with ribosomes is due to adsorption the substantial release of enzyme from the ribosomes by treatment with puromycin supports the view that some at least of the enzyme associated with ribosomes represents nascent protein. The association of enzyme with the ribosomes from a broad spectrum of size of polysomes was unexpected but is in close accord with that found by Warren & Peters (1965) for nascent chains of albumin. It is probably to be accounted for by the random breakdown during preparations of a polycistronic messenger RNA. Therefore we believe that there is substantial evidence that the isolated polysomes effect the synthesis of reductase. For the reasons already given in the Results section we consider that the results obtained for the reductase are typical for at least a group of membrane proteins.

For the preparation of the various polysomes every care was taken to ensure that the separation of bound and free polysomes was as complete as possible. Bloemendal, Bont & Benedetti (1964) concluded from their studies that free-polysome preparations represent ribosomes, which, in the cell, either were not associated with the membrane of the endoplasmic reticulum or were bound to these membranes by much weaker forces than those involved in binding of the so-called 'bound' polysomes. The free and bound polysome preparations therefore probably originate from two distinct kinds of cellular ribosomes. In contrast the deoxycholate-treated polysomes (C-ribosomes) are derived from the total polysome population of the cell. From the results given in Table 2 it is clear that all kinds of polysomes were able to effect the synthesis of the reductase. Although it has been shown by Bloemendal *et al.* (1964) that preparations of free polysomes are able to incorporate amino acids into protein, the present work is the first demonstration of the synthesis of a specific protein by this fraction. Moreover, it is of interest that the detergent-treated polysomes were also able to effect such a synthesis, albeit at a lower rate than the other fractions, for in the past such preparations have been shown to be active for the incorporation of amino acid but unable to synthesize a specific protein (see, e.g. Decken & Campbell, 1962).

The above results do not support the idea of two functionally separate groups of polysomes in the liver cell. However, we have been concerned only with the activity of the polysomes for the synthesis of membrane proteins. That bound polysomes play a major role in the synthesis of proteins for export has been previously demonstrated (Sargent & Campbell, 1965; Lawford & Schachter, 1966; Redman, 1968).

Naturally, the synthesis of membrane protein by both the bound and free polysomes is relevant to theories concerning the biogenesis of the membranes of the endoplasmic reticulum. It is clear from the current experiments that either 'free polysomes' or polysomes treated with deoxycholate can effect the synthesis of a constitutive membrane protein. The newly synthesized protein present in the incubation supernatant is bound by added smooth endoplasmic reticulum. This finding supports the hypothesis that predicts that the enzyme profile of the endoplasmic reticulum can be varied independently of the rough endoplasmic reticulum. This idea could be extended to include the constituents of other membranes of the cell. Furthermore the newly synthesized reductase seems to have an avidity for the ribosomes and this may be of importance in the synthesis of membrane protein.

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