Biochem. J. (1974) **140**, 157–167
Printed in Great Britain

Role of Membrane-Bound and Free Polyribosomes in the Synthesis of Cytochrome *c* in Rat Liver

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(*Received* 29 *October* 1973)

The functional distinction of membrane-bound and free polyribosomes for the synthesis of exportable and non-exportable proteins respectively is not so strict as was initially thought, and it was therefore decided to investigate their relative contribution to the elaboration of an internal protein integrated into a cell structure. Cytochrome *c* was chosen as an example of a soluble mitochondrial protein, and the incorporation of [^{14}C]leucine and δ-amino[^{14}C]laevulinate into the molecule was studied by using different ribosomal preparations from regenerating rat liver. A new procedure was devised for the purification of cytochrome *c*, based on ion-exchange chromatography combined with sodium dodecyl sulphate–polyacrylamide-gel electrophoresis. In spite of cytochrome *c* being a non-exportable protein, the membrane-bound polyribosomes were at least as active as the free ribosomes in the synthesis *in vitro* of the apoprotein and the haem moiety. The detergent-treated ribosomes could also effect the synthesis of cytochrome *c*, although at a lower rate. Since in liver more than two-thirds of the ribosomes are bound to the endoplasmic-reticulum membranes, it is considered that *in vivo* they are responsible for the synthesis of most of the cytochrome *c* content of the cell. This suggests that in secretory tissues the endoplasmic reticulum plays a predominant role in mitochondrial biogenesis, although free ribosomes may participate in the partial turnover of some parts of the organelle. The hypothesis on the functional specialization of the different kinds of ribosomes was therefore modified to account for their parallel intervention in the synthesis of proteins associated with membranous structures.

Membrane-bound polyribosomes are present mainly in the secretory tissues, and therefore it is natural to assume that such ribosomes are responsible for the synthesis of proteins for export, since the endoplasmic-reticulum membranes and vesicles would facilitate the extracellular transport of nascent protein. Conversely, free ribosomes are more abundant in rapidly growing tissues and non-secretory tissues, and are thought to specialize in the elaboration of proteins for the internal needs of the cell (see, e.g., Campbell, 1970; Tata, 1971). Different authors (Redman, 1969; Hicks *et al.*, 1969; Takagi & Ogata, 1968; Ganoza & Williams, 1969) showed that albumin and other serum proteins are synthesized by membrane-bound ribosomes, whereas ferritin is elaborated by free ribosomes. However, this functional differentiation was not as clear as was first proposed, since Ragnotti *et al.* (1969) concluded that both kinds of ribosomes were active in the synthesis of the same non-exportable protein, NADPH–cytochrome *c* reductase. Other indirect evidence (Dallner *et al.*, 1966; Omura & Kuriyama, 1971) suggested that certain proteins, integrated in cell membranes, were manufactured by membrane-bound ribosomes. Another discrepancy was that cells such as HeLa cells, in spite of their not exporting proteins, do contain a rough endoplasmic reticulum (Attardi *et al.*, 1969).

Uenoyama & Ono (1972) have demonstrated that in rat hepatoma 5123, where albumin is not exported, only free polyribosomes are responsible for the elaboration of this protein. In normal cells membrane-bound ribosomes have been shown to be the sole site for the synthesis of serum albumin (Takagi & Ogata, 1971; Tanaka & Ogata, 1972), β-lactoglobulin (Gaye & Denamur, 1970), and thyroglobulin (Vassart, 1972; Vassart & Dumont, 1973). In other cases one ribosomal class was merely much more active for the synthesis of a particular protein than the other class. Such results are interpreted as demonstrating that the protein is preferentially elaborated *in vivo* by the most active class of ribosomes. Thus it is concluded that the following proteins are synthesized by membrane-bound ribosomes: immunoglobulins (Lisowska-Bernstein *et al.*, 1970; Scherr & Uhr, 1971), β-lactalbumin (Brew & Campbell, 1967) and glycoproteins (Hallinan *et al.*, 1968), whereas free ribosomes synthesize ferritin (Redman,

1969; Hicks *et al.*, 1969; Puro & Richter, 1971), myosin (Nihei, 1971) and the brain-specific protein S-100 (Amaldi *et al.*, 1973).

Besides NADPH–cytochrome *c* reductase, two other proteins, catalase (Higashi *et al.*, 1972; Kashiwagi *et al.*, 1971) and globin (Woodward *et al.*, 1973), are similarly synthesized by free and membrane-bound ribosomes, although in the reticulocyte the latter are not attached to an endoplasmic reticulum but to the cell membrane. Therefore only the relative proportions in the cell of both kinds of ribosomes would determine their contribution to the formation of those proteins *in vivo*.

The mitochondrial proteins are synthesized for the internal needs of the cell, but they are also integrated into membrane structures which might have some biogenetic interrelationship with the endoplasmic reticulum. As more than 90% of the mitochondrial proteins are synthesized outside the organelle (Hawley & Greenawalt, 1970), the question arises as to which class of cytoplasmic ribosomes is responsible. To investigate this problem we have selected as a model the synthesis *in vitro* of an important constituent of the electron-transport chain, cytochrome *c*, by different subcellular fractions from rat liver (González-Cadavid *et al.*, 1971). In the present paper we demonstrate that membrane-bound, free and detergent-treated ribosomes can effect the synthesis of this protein, although *in vivo* cytochrome *c* is probably formed mainly in the rough endoplasmic reticulum.

Materials and Methods

Chemicals

L-[U-^{14}C]leucine (sp. radioactivity 297mCi/mmol) and δ-amino[^{14}C]laevulinic acid hydrochloride (sp. radioactivity 59mCi/mmol) were purchased from the Commissariat à L'Energie Atomique (CEA), Gif-sur-Yvette, France. Sucrose, *NNN'N'*-tetramethylethylenediamine, acrylamide, hexane (boiling range 60–80°C) and glycerol were from BDH Chemicals Ltd., Poole, Dorset, U.K., AnalaR grade whenever available. The ethylene glycol diacrylate (ethylene diacrylate) was from K & K Laboratories, Inc., Plainview, N.Y., U.S.A. The Insta-gel emulsifier was from Packard Instruments Co., Downers grove, Ill., U.S.A., and the Liquifluor scintillator was from Amersham/Searle Corp., Des Plaines, Ill., U.S.A. All other materials were as specified by González-Cadavid *et al.* (1971).

Animals

Sprague–Dawley rats from a closed colony bred at the Instituto de Medicina Experimental, Universidad Central de Venezuela, Caracas, Venezuela, were used throughout. Partial hepatectomy was performed by the technique described by Higgins & Anderson (1931) on male rats (body wt. 150±10g), and liver regeneration was allowed to proceed for 48h. For other details see González-Cadavid *et al.* (1971).

Preparation of subcellular fractions

All operations were performed at 2–3°C. One or several regenerating livers were used, as specified in each experiment. The microsomal and cell-sap fractions were prepared in medium B, containing 10mM-$MgCl_2$, 35mM-Tris–HCl buffer, pH7.8, at 20°C, 25mM-KCl and 0.15M-sucrose, as indicated by González-Cadavid *et al.* (1971), except that the ratio ml of medium/g of liver was 2.5 instead of 2. In most experiments, however, the cell-sap fraction was obtained separately by centrifuging the post-mitochondrial supernatant at 165000*g* for 60min and removing the residual ribosomes by ultrafiltration through a 0.45μm Millipore filter (HAWP 04700). The pH5 fraction was prepared from 5ml of cell sap by adjusting the pH to 5.2 with 1M-acetic acid. The resulting precipitate was collected by centrifugation at 3000*g* for 5min and suspended in 1ml of 0.5M-Tris–HCl, pH7.8, followed by the addition of 4ml of medium B. The Sephadex-treated cell-sap fraction was obtained from the cell sap by removing amino acids and low-molecular-weight factors through a column of Sephadex G-25 equilibrated with medium B.

The membrane-bound, free and detergent-treated total polyribosomes (C-ribosomes) were prepared as described by Ragnotti *et al.* (1969), with 2.0M-sucrose as the bottom layer. The microsomal and ribosomal fractions were obtained freshly for each experiment, whereas the soluble fractions were prepared for several experiments and stored in batches at −60°C. Each portion was thawed only once and the excess discarded.

Incubation conditions

The pellets were gently suspended in medium B, and the RNA concentration determined from its E_{260} in a 10μl sample diluted to 1ml with 0.5% sodium dodecyl sulphate in medium B, by using the correction factors proposed by Ragnotti (1971). The RNA concentration was then adjusted to 1.25mg/ml. Incubations were performed for 30min at 37°C, in a total volume of 0.5ml containing the following: RNA (0.25mg/ml); cell sap [3–3.5mg of protein/ml as determined by the procedure of Lowry *et al.* (1951)]; Sephadex-treated cell sap (2–2.5mg of protein/ml) or pH5 fraction (0.8–1.2mg of protein/ml); sucrose (90mM); Mg^{2+} (6mM); K^+ (25mM); Tris–HCl buffer, pH7.8 at 20°C (21mM. or 39mM when the pH5 fraction was used); ATP (2mM); phosphoenolpyruvate (10mM); pyruvate kinase

(50μg/ml); GTP (0.25mM); and radioactive precursor as indicated. The tubes were shaken in a metabolic incubator at approx. 100 oscillations/min. The incorporation of radioactivity was stopped by immersing the tubes in an ice bath and adding the corresponding non-radioactive compound to a final concentration of 10mM. When the incubations were carried out for the study of the synthesis of cytochrome *c*, the total volume was 5ml in 50ml Erlenmeyer flasks. Variations to this basic technique are indicated for each experiment.

Extraction and initial purification of cytochrome c

A new simplified procedure based on technique no. 2 of González-Cadavid *et al.* (1971) was applied as follows. Horse heart cytochrome *c* (0.2ml of a 3mg/ml solution) was added as carrier to the incubation suspension and the volume was diluted to 15ml with water. All the operations were performed at 0–2°C. The pH was adjusted to 3.8–4.0 with 0.125M-H_2SO_4 and left for 30min with occasional stirring. After centrifugation at 31500*g* for 15min, the supernatant was kept and the pellet was homogenized in 1ml of 1M-NaCl and ultrasonicated at 0°C in a MSE 100W ultrasonic disintegrator at 8.5μm amplitude, four times for 15s each. Centrifugation was repeated and the supernatant was pooled with the pH4 extract. After neutralization, $(NH_4)_2SO_4$ was added up to 60% saturation at 0°C with continuous stirring.

The precipitate was discarded by centrifugation at 31500*g* for 15min, and the supernatant was dialysed against four changes of 20mM-ammonium acetate for 48h. The dialysis tubing was previously boiled for 30min in 1% $NaHCO_3$. The solution was oxidized with $K_3Fe(CN)_6$ (0.5mM final concn.) and passed through a column (1cm×4cm) of Amberlite CG-50, equilibrated with 0.25M-K_2HPO_4 buffer at pH7. The column was washed with 30ml of 0.125M-K_2HPO_4 buffer, pH7, containing 0.5mM-$K_3Fe(CN)_6$, and with 3ml of water. The band of cytochrome *c* was eluted with 1M-K_2HPO_4 and the amount measured from the absorption at the isosbestic point, 410nm ($E^{1\%}_{1cm}$ 86.6). $(NH_4)_2SO_4$ and trichloroacetic acid were then added to 80% saturation and pH3.5 respectively, and the precipitate was sedimented at 31500*g* for 15min.

To eliminate the $(NH_4)_2SO_4$, which affects the solubility of cytochrome *c*, the pellet was dissolved in 2.5ml of water, and 50% (w/v) trichloroacetic acid was added to a final concentration of 5% (w/v). The precipitate was again centrifuged and dissolved in 1ml of 10% (w/v) acetic acid; then 20ml of acid acetone (1ml of 2.5M-H_2SO_4 for 100ml of acetone) was added and the precipitate sedimented at 31500*g* for 15min. The sequential treatment with acetic acid and acid acetone was repeated once and the sediment was finally suspended in 0.3ml of 10% (v/v) acetic acid.

Final purification of cytochrome c by polyacrylamide-gel electrophoresis

Hereafter all the steps were performed at room temperature. The cytochrome *c* was dialysed against four changes of 0.01M-Na_2HPO_4 (pH7.2)–0.1% sodium dodecyl sulphate–5mM-EDTA during 48h, which completely dissolved the haemoprotein. A 20μl portion was used for spectrophotometric measurement, after dilution to 1ml with dialysis buffer, and then counted for radioactivity. Sucrose was added to the remainder of the sample (final concn. 20%, w/v) and it was layered on 0.85cm×5cm gels made in tandem with 10% acrylamide in the top 2cm and 15% acrylamide in the bottom. The other ingredients of the gel were 0.25% ethylenediacrylate, 10% (v/v) glycerol, 0.05% *NNN'N'*-tetramethylethylenediamine, 0.075% ammonium persulphate, 0.1M-Na_2HPO_4, pH7.2, and 0.1% sodium dodecyl sulphate. The gels had been pre-run for 30min at 2mA/tube. Samples were introduced into the gels at 3mA/tube and the electrophoresis was carried out at 5mA/tube for 14–15h (anode in bottom chamber). The cytochrome *c* band was located at about 36mm, with a width of 3–4mm.

Determination of radioactivity of the cytochrome c samples

After electrophoresis the gels were frozen with solid CO_2 in hexane, and cut into 1mm sections with an electronically controlled gel slicer made by Metaloglass Inc., Boston, Mass., U.S.A. Owing to the stretching of the gel, the number of slices was usually about 55. Each slice was put in a counting vial and 0.2ml of a freshly prepared mixture of H_2O_2 (120-volume) and aqueous conc. NH_3 (99:1, v/v) was added (Goodman & Matzura, 1971). The vials were left at 37°C overnight and 4ml of Insta-gel was then added. In certain cases only the cytochrome *c* band was sectioned, a total of seven slices being collected, which were put together in a single vial with 1ml of H_2O_2–NH_3, requiring 12ml of Insta-gel for radioactivity counting. All vials were left at 4°C in the darkness for at least 3h to eliminate some occasional spurious counts decaying exponentially with time. The determinations of radioactivity were performed in an automatic liquid-scintillation spectrometer (model 720, Nuclear-Chicago Corp.) to a 3% statistical error at 68% probability. Efficiencies were 64 and 78%, respectively, for the 4 and 12ml samples, as calculated by internal standardization with [^{14}C]toluene.

The diluted samples used for the determination of cytochrome *c* immediately before electrophoresis

(1ml) were counted for radioactivity with 5ml of the scintillation fluid T21 proposed by Patterson & Greene (1965), containing 2vol. of toluene, with 0.4% of 2,5-diphenyloxazole and 0.01% of 1,4-bis-(5-phenyloxazol-2-yl)benzene, and 1 vol. of Triton X-100. Efficiency was 74%.

Determination of radioactivity in the total protein samples

Samples (50μl) from the incubation suspensions were adsorbed on to glass-fibre discs (24mm diam.) and processed by a modification of the method of Mans & Novelli (1961), including successive washings with 10ml each of 10% (w/v) trichloroacetic acid (containing 0.25mg of L-leucine/ml or 0.1mg of δ-aminolaevulinic acid/ml), 5% (w/v) trichloroacetic acid (twice at room temperature, once at 90°C for 15min and once at room temperature, in that order), ethanol–ether (3:1, v/v) (twice), acetone, acid acetone (to remove any non-covalently linked haem), and ether (twice).

The glass-fibre discs were counted for radioactivity in vials with 2ml of a diluted Liquifluor, containing 0.4% of 2,5-diphenyloxazole and 0.05% of 1,4-bis-(5-phenyloxazol-2-yl)benzene in toluene, to a 1% statistical error at 68% probability. The efficiency was 78%.

Results

Incubation conditions for membrane-bound and free polyribosomes

To get satisfactory incorporation of radioactivity into cytochrome *c* it was essential to define adequate conditions for the subcellular system *in vitro*. These were chosen from the ones used previously for the microsomal fraction (González-Cadavid *et al.*, 1971), which were based on the work of Ragnotti *et al.* (1969) on the synthesis of NADPH–cytochrome *c* reductase by membrane-bound ribosomes. The basic ionic medium supplemented with an energy source was used initially for testing the ability of different soluble fractions to support protein and haem synthesis *in vitro* by membrane-bound polyribosomes. Table 1 shows that the cell sap passed through Sephadex G-25 gave a higher incorporation of [^{14}C]leucine into protein than did the pH5 fraction, and the least active was the untreated cell sap. The incorporation of a haem precursor, δ-aminolaevulinate, into an acid-acetone-resistant link to protein was more than 10-fold higher with cell sap than with the pH5 fraction, and the Sephadex-treated cell sap had an intermediate activity.

The effect of varying the amounts of rRNA was investigated with the three different kinds of soluble

Table 1. *Protein synthesis in vitro by membrane-bound polyribosomes*

The membrane-bound ribosomes and soluble fractions were prepared and incubated as described in the text, except that concentrations were as follows: RNA, 150μg/ml; [^{14}C]leucine, 0.25μCi/ml; δ-amino[^{14}C]laevulinate, 0.20μCi/ml. Values are means of triplicate determinations.

	Total radioactivity of the proteins in the incubation/mg of RNA (d.p.m./mg of RNA)	
Soluble fraction	[^{14}C]Leucine	δ-Amino[^{14}C]-laevulinate
pH5 fraction	49950	19205
Cell sap	15690	253230
Sephadex-treated cell sap	65915	96980

fractions. A considerable decrease in protein radioactivity per mg of RNA in the system incubated with [^{14}C]leucine and Sephadex-treated cell sap was observed for RNA concentrations up to about 200μg/ml, where a plateau was reached (Fig. 1*a*), which resembles the results of Ragnotti (1971) obtained with the microsomal fraction and detergent-treated ribosomes. The difference in activity between this system and the ones in the presence of pH5 fraction or cell sap was more marked at lower RNA concentrations (Fig. 1*b*). At 250μg of RNA/ml, the incorporation of [^{14}C]leucine with the gel-filtered cell sap was three-fold higher than with the untreated cell sap, and about 50% higher than with the pH5 fraction. Therefore as a routine the cell sap was passed through Sephadex G-25 for the incubations with [^{14}C]leucine, and the RNA concentration was fixed at 250μg/ml, since the total radioisotope incorporated per ml of medium was higher than at lower RNA concentrations and the specific radioactivity was reasonable. The incubations with δ-amino[^{14}C]-laevulinate were carried out at the same ribosomal concentration, but with untreated cell sap, which was the more active soluble fraction (Table 1). A similar criterion was applied to systems with other particulate fractions, such as free polyribosomes, C-ribosomes and microsomal preparations. The exceptions to these procedures are indicated in the appropriate experiments.

Fig. 2 shows that for a relatively low RNA concentration (40μg/ml) an increase in added [^{14}C]-leucine was not followed by a proportional increment in the protein radioactivity. This indicates that the free leucine pool is small, and can be estimated for this particular experiment as around 25–30nmol/mg of RNA. The amount of leucine incorporated into

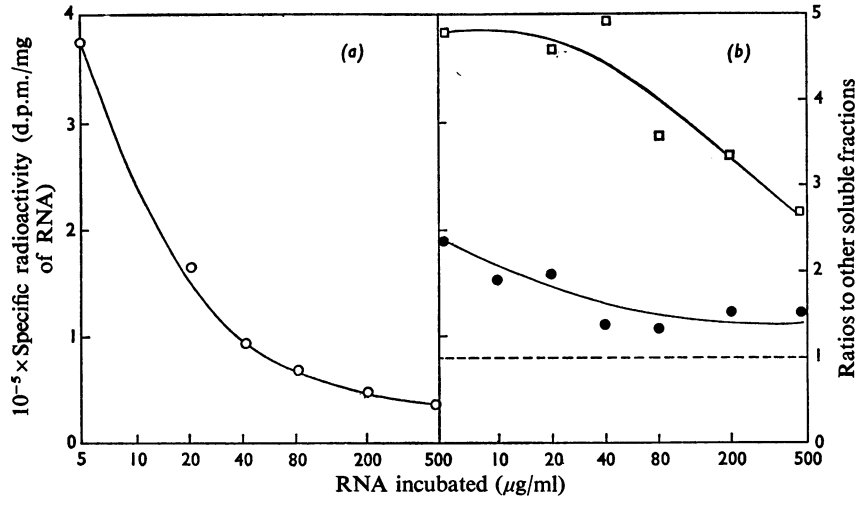

Fig. 1. *Effect of RNA concentration on protein synthesis by membrane-bound polyribosomes*

The subcellular fractions were prepared and incubated as described in the text, except that concentrations were: RNA, as specified in the Figure; [^{14}C]leucine, 0.20μCi/ml. Each determination was performed in duplicate. (*a*) Total radioactivity of the proteins in the system incubated with Sephadex-treated cell sap is expressed/mg of rRNA; (*b*) ratios of incorporation in that system compared with the systems incubated with cell sap (□) or pH5 fraction (●). Note the logarithmic scale.

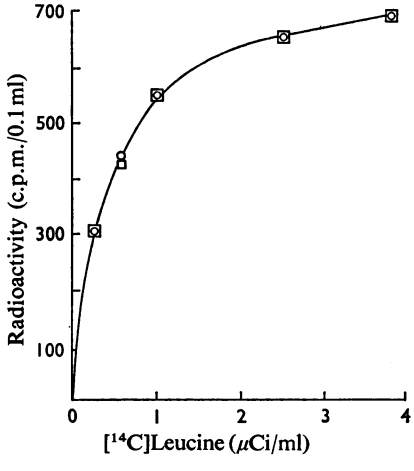

Fig. 2. *Effect of* [^{14}C]*leucine concentration on protein synthesis by membrane-bound polyribosomes in the presence of Sephadex-treated cell sap*

The subcellular fractions were prepared and incubated as described in the text, except that concentrations were: RNA, 40μg/ml; [^{14}C]leucine, as specified in the Figure. Each determination was performed in duplicate. ○, Experimental points; □, curve calculated from two of the experimental points by assuming dilution of the added radioactivity by an endogenous pool of leucine.

protein was 300nmol/mg of RNA. Assuming that part of the leucine pool is associated with the particulate fraction, in the experiments with cytochrome *c* the concentration of [^{14}C]leucine had to be increased to compensate for the higher ribosomal RNA concentration.

The membrane-bound ribosomes in the presence of the Sephadex-treated cell sap showed essentially the same features of the microsomal fraction+pH5 fraction system for protein synthesis (Table 2). No incorporation was observed at 0°C or by removing the ribosomes or the energy source. A considerable residual activity was left in the absence of the soluble fractions, probably as a consequence of the adsorption of RNA and aminoacetyl-synthetases to the particulate fractions. The effect of chloramphenicol was negligible, whereas puromycin and emetine blocked protein synthesis by both systems. Cycloheximide was active only at high concentrations, but this has also been observed previously with non-initiating systems (see Pestka, 1971).

Synthesis of cytochrome c by membrane-bound and free polyribosomes

As our previous procedures for the isolation and purification of cyrochrome *c* were based mainly on ion-exchange chromatography (González-Cadavid *et al.*, 1971), we decided to replace some of these steps

Table 2. *Effect of inhibitors on protein synthesis by the ribosomal systems*

The particulate fractions were obtained and assayed in separate experiments, as described in the text, except that the soluble fractions were as indicated below, and the concentrations were as follows: RNA, 250 μg/ml; [^{14}C]leucine, 0.25 μCi/ml. Control values of total radioactivity of the proteins in the incubation were 31440 (membrane-bound ribosomes) and 70290 (microsomal fraction) d.p.m./mg of RNA. Values are averages of two separate experiments for each system, with determinations performed in triplicate.

Modification of basic system	Inhibition of incorporation relative to basic system (%): Microsomal fraction+ pH5 fraction	Membrane-bound ribosomes+ Sephadex-treated cell sap
(1) Zero-time incubation	98	98
(2) –Particulate fraction	99	96
(3) –Soluble fraction	32	51
(4) –Energy	99	96
(5) +Chloramphenicol (50 μg/ml)	7	3
(6) +Emetine (50 μg/ml)	85	—
(7) +Emetine (100 μg/ml)	90	78
(8) +Cycloheximide (100 μg/ml)	35	19
(9) +Cycloheximide (200 μg/ml)	53	—
(10) +Cycloheximide	71	—
(11) +Puromycin (1 mM)	95	92

by polyacrylamide-gel electrophoresis, to introduce another purity criterion and simplify the technique. Preliminary experiments showed that a tandem gel made of 10% acrylamide in the top and 15% in the remainder gave the best separation of cytochrome *c*, as compared with plain 5, 10 and 15% gels or combinations of these concentrations. The electrophoresis was done in the presence of sodium dodecyl sulphate to avoid any possible complexing of cytochrome *c* with other proteins. Mercaptoethanol, which is usually added to separate protein subunits by breaking disulphide links, cleaves the thioether bridge between the apoprotein and prosthetic group and had therefore to be omitted. Initially, we attempted to run the proteins precipitated by trichloroacetic acid from the incubations, but the radioactivity profile showed a considerable overlapping of bands. The application of the extraction and salting-out steps of the procedure were not enough to eliminate this background, and only when dialysis and ion exchange were introduced, was the electrophoretic pattern acceptable.

Incubation of membrane-bound and free polyribosomes with Sephadex-treated cell sap and [^{14}C]leucine for investigation of the synthesis of cytochrome *c* was carried out in systems scaled-up by a factor of 10 (5ml). Dithioerythritol, haemin and acetylglycine were added to reproduce the conditions used previously (González-Cadavid *et al.*, 1971). Fig. 3 shows the radioactivity profiles obtained by applying sodium dodecyl sulphate–polyacrylamide gel electrophoresis to the purified cytochrome *c* fractions, and clearly the position of the haemoprotein band coincides with a peak of radioactivity. The incorporation was higher in the membrane-bound that in the free ribosomes. A thin coloured band migrating more slowly than the main band was also labelled. Runs with pure horse heart cytochrome *c* showed this satellite band, which is probably a denatured polymeric form of the carrier cytochrome *c*. When a similar concentration of δ-amino[^{14}C]-laevulinate was used for labelling the acid-acetone-resistant haem moiety of cytochrome *c*, the radioactivity was about threefold higher than with [^{14}C]leucine and the peak was quite sharp (Fig. 4). Again the membrane-bound ribosomes were more active than the free ribosomes. In two other experiments, not illustrated here, the results with the free ribosomes were approximately similar, whereas the membrane-bound ribosomes had lower activity. As a consequence, one can conclude that both kinds of polyribosomal populations can carry out the synthesis of cytochrome *c*, membrane-bound ribosomes being slightly more active on average than the free ones (Table 3), paralleling the situation with total proteins. Many authors have found that the protein-synthetizing activity of the microsomal or membrane-bound ribosomal fractions is lower than that shown by membrane-free or detergent-treated polyribosomes [see Ragnotti (1971) for extensive list of references], but there is also conflicting evidence (Andrews & Tata, 1971; Murthy, 1972*a*; Venkatesan & Steele, 1972; see also references quoted by MacDonald & Korner, 1971).

Re-examination of the conditions for the synthesis of cytochrome c in vitro by the microsomal fraction

Since in the experiments just described supplementation with dithioerythritol, acetylglycine and haemin was done only with [^{14}C]leucine and not with δ-amino[^{14}C]laevulinate the question arose as to whether these compounds really favoured the synthesis of cytochrome *c* as had been assumed in our previous work but not tested (González-Cadavid *et al.*, 1971). We now investigated this point by incubating the particulate fraction that was easiest to obtain, i.e. the microsomal fraction, with δ-amino[^{14}C]aminolaevulinate, under different conditions. Table 4 shows that the basic system in the presence of cell sap is much more efficient than the membrane-bound ribosomes for the incorpora-

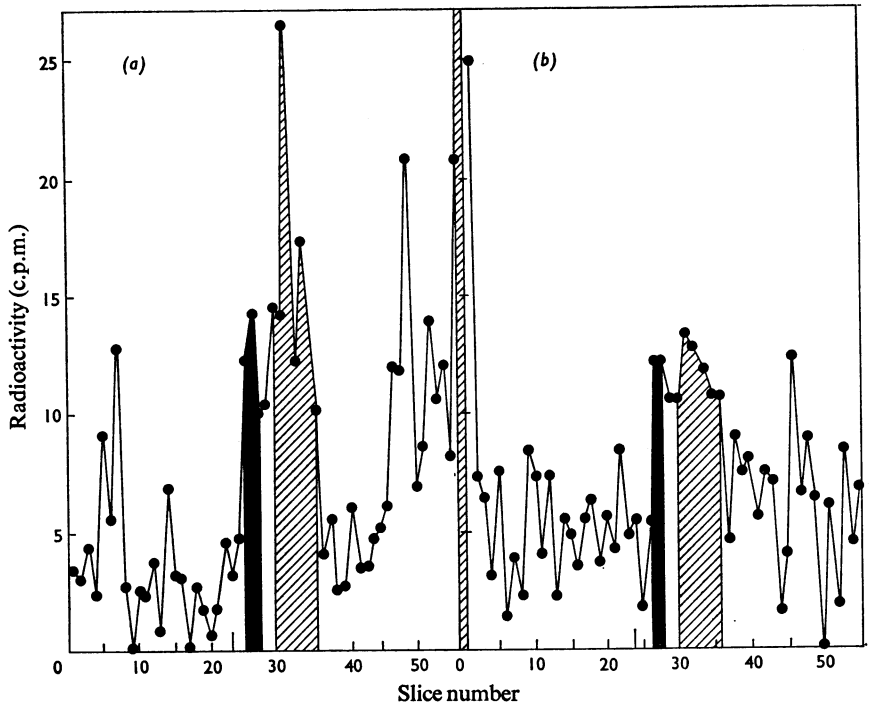

Fig. 3. *Electrophoretic profile of a cytochrome c fraction after the incubation of membrane-bound and free polyribosomes with [^{14}C]leucine*

The subcellular fractions were prepared and incubated as described in the text, except that: RNA concentration was 250μg/ml; Sephadex-treated cell sap was used (2.5mg of protein/ml); [^{14}C]leucine concentration was 6μCi/ml; acetylglycine (2.5mM), dithioerythritol (1mM) and haemin (0.1mM) were added; total volume was 5ml. Cytochrome *c* was extracted and purified as indicated in the text, including the sodium dodecyl sulphate–polyacrylamide-gel electrophoresis. (*a*) Membrane-bound polyribosomes; (*b*) free polyribosomes. Hatched area, main cytochrome *c* band; black area, satellite cytochrome *c* band.

tion of δ-aminolaevulinate into an acid-acetone-resistant linkage, both in total protein (four–fivefold higher) and in cytochrome *c* (threefold higher). This is probably a consequence of the much longer procedure required for the preparation of the membrane-bound polyribosomes, which would allow the action of the ribonucleases located in the endoplasmic-reticulum membrane, particularly as the inhibitors present in the cell sap remain on top of the sucrose gradient.

The removal of the energy source or the microsomal fraction did not completely inhibit the incorporation into cytochrome *c*, demonstrating that besides the energy-dependent ribosomal-located process, there is a considerable labelling not related to protein synthesis. A first assumption is that a pool of free apoprotein would be linked to the prosthetic group synthesized by the system. Either haem formation or its linkage to cytochrome *c* apoprotein require factors present in the cell sap, since when the cell sap was omitted from the medium practically no incorporation occurred. The labelling of cytochrome *c* was inhibited by 78% by emetine, thus confirming its dependence from protein synthesis. The addition of dithioerythritol impaired the activity, and the same occurred when the medium was supplemented with this thiol-group-protecting reagent, acetylglycine and amino acids.

The incorporation of δ-amino[^{14}C]laevulinate into an acid-acetone-resistant linkage to total protein is probably mainly a result of an unspecific binding of haem to already synthesized proteins, rather than a process truly dependent on protein synthesis, since the omission of the energy source or the addition of emetine did not lower the protein radioactivity. Whereas the cell sap is essential for the incorporation, the omission of ribosomes left a considerable residual labelling in total protein.

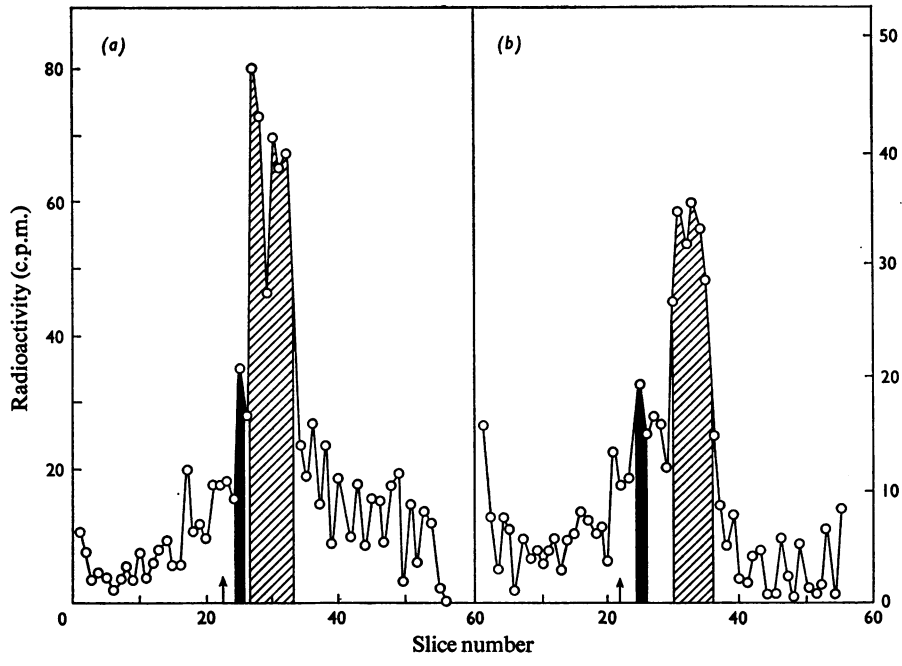

Fig. 4. *Electrophoretic profile of a cytochrome c fraction after the incubation of membrane-bound and free polyribosomes with δ-amino[^{14}C]laevulinate*

Conditions were identical with those of Fig. 3, except that δ-amino[^{14}C]laevulinate (6 μCi/ml) was used instead of [^{14}C]leucine. (*a*) Membrane-bound polyribosomes; (*b*) free polyribosomes. Hatched area, main cytochrome *c* band; black area, satellite cytochrome *c* band.

Table 3. *Synthesis of cytochrome c by membrane-bound and free polyribosomes*

Three separate experiments were carried out under conditions identical with those of Figs. 4 and 5. The incorporation into cytochrome *c* was calculated by adding the d.p.m. of the main band, and finally correcting for the individual recovery of carrier cytochrome *c* throughout the whole procedure, excluding the electrophoresis itself (mean of 37%). Values are averages of the three experiments.

	Total radioactivity of the proteins in the incubation/mg of RNA (d.p.m./mg of RNA)	Radioactivity in cytochrome *c*	
		Total in incubation (d.p.m.)	Relative to input in gel (%)
[^{14}C]Leucine			
(a) Membrane-bound	86840	287	23
(b) Free	67500	257	23
Ratio (a)/(b)	1.29	1.12	—
δ-Amino[^{14}C]laevulinate			
(a) Membrane-bound	147260	946	41
(b) Free	132240	935	43
Ratio (a)/(b)	1.11	1.01	—

Synthesis of cytochrome c by detergent-treated ribosomes

One of the main objectives in investigating the synthesis *in vitro* of cytochrome *c* was to obtain a system that could reflect the variations *in vivo* in the rate of formation of this protein, allowing in turn a study of its regulation at the level of translation and/or transcription. The first condition for such a system is reproducibility from animal to animal, to establish an accurate comparison between experimental and control rats. Table 5 shows that the

Table 4. *Incorporation of δ-amino[^{14}C]laevulinate into cytochrome c by the microsomal fraction under different conditions*

The microsomal, cell sap and pH 5 fractions were obtained and incubated as described in the text, except that RNA concentration was 250 μg/ml; δ-amino[^{14}C]laevulinate concentration was either 4 μCi/ml (Expt. 1) or 2 μCi/ml (Expt. 2); when the systems were supplemented as indicated below, the final concentrations were: acetylglycine (2.5 mM), dithioerythritol (1 mM), emetine (100 μg/ml), and amino acid mixture (all protein amino acids, except leucine, cystine, hydroxyproline and hydroxylysine; 0.05 mM each); total volume was 5 ml. Cytochrome *c* was extracted and purified as detailed in the text, and the polyacrylamide-gel slices corresponding to the main cytochrome *c* band were pooled, dissolved and counted for radioactivity in a single vial as described.

Additions or omissions	Total radioactivity of the proteins in the incubation/mg of rRNA (d.p.m./mg of RNA)	Radioactivity in cytochrome *c*		
		Total in incubation (d.p.m.)	Referred to: Control value (%)	Referred to: Input in gel (%)
Expt. 1 (4 μCi/ml)				
Basic system	644250	2500	100	37
−Energy	884200	1335	53	34
−Microsomal fraction	122015	940	38	36
−Cell sap	27190	106	4	21
+Emetine	678540	550	22	12
+Dithioerythritol	654270	1525	61	69
Expt. 2 (2 μCi/ml)				
Basic system	382505	665	100	34
+Dithioerythritol, acetylglycine and amino acids	638785	430	64	10

Table 5. *Reproducibility of the synthesis of cytochrome c in vitro by cytoplasmic ribosomes*

The microsomal fractions were prepared from four rats and the detergent-treated total polyribosomes from another four as described in the text. All ribosomal suspensions were processed and incubated separately with cell sap under conditions indicated in the text, except that: RNA concentration was 250 μg/ml; δ-amino[^{14}C]laevulinate concentration was 2 μCi/ml; the basic system was supplemented with an amino acid mixture (0.05 mM each) and acetylglycine (2.5 mM); total volume was 5 ml. Cytochrome *c* was processed as indicated in Table 4.

Fraction incubated	Total radioactivity of the proteins in the incubation/mg of rRNA (d.p.m./mg of RNA)	Radioactivity in cytochrome *c*	
		Total in incubation (d.p.m.)	Referred to input in gel (%)
Microsomal fraction			
Rat 1	304560	1246	—
Rat 2	338965	2047	55
Rat 3	370350	2070	52
Rat 4	399770	1134	51
Average	353410	1624	53
Detergent-treated total polyribosomes			
Rat 5	260120	571	—
Rat 6	250410	552	12
Rat 7	260055	701	25
Rat 8	318110	581	17
Average	272170	601	18

incorporation of δ-amino[^{14}C]laevulinate into total protein with microsomal fractions from four different livers was relatively constant, with a range of only 13% around the mean. The labelling of cytochrome *c* was, however, much more variable, with a range of 30% around the mean, and 70% between two pairs of animals.

The detergent-treated total polyribosomes, as suspected from the results with free polyribosomes, were able to synthesize cytochrome *c*, and the rate was

comparable if allowance is made for the differences in the concentration of δ-amino[^{14}C]laevulinate. The reproducibility was much better than with the microsomal fraction and the range was 15% from the mean.

Discussion

The main conclusion of the present work is the demonstration that membrane-bound, free and detergent-treated total ribosomes can carry out the synthesis of cytochrome *c*. This confirms and extends our previous findings (González-Cadavid *et al.*, 1971), by showing that the incorporation of radioactive precursors of the apoprotein and prosthetic group of cytochrome *c* by the microsomal fraction is due to both kinds of its constituents, rough endoplasmic reticulum and free ribosomes, and not to either of them exclusively. The role of membrane-bound ribosomes in this process had been subsequently shown by González-Cadavid *et al.* (1972), but no similar incorporation by the free and detergent-treated ribosomes was detected. Now, using different procedures of incubation and purification, we have obtained a reasonable labelling of the whole cytochrome *c* molecule by the ribosomes alone, irrespective of whether the membrane was already absent *in vivo* or was artificially eliminated *in vitro*. The lower activity of C-polyribosomes coincides with the results obtained by Ragnotti *et al.* (1969) for NADPH–cytochrome *c* reductase.

The fact that both membrane-bound and free ribosomes have similar activity in the synthesis of the same protein is in agreement with findings of other authors (Ragnotti *et al.*, 1969; Higashi *et al.*, 1972; Kashiwagi *et al.*, 1971; Woodward *et al.*, 1973). The increase in the synthesis of proteins for the internal needs of the cell occurring after partial hepatectomy (Zweig & Grisham, 1971) or body exposure to ionizing radiation (Ekren & Yatvin, 1972) is not accompanied by a change in the ratio of free to membrane-bound polyribosomes. These findings suggest that the functional differentiation of both kinds of ribosomes is not as simple as was first supposed.

In particular, our own data indicate that cytochrome *c* is mainly elaborated by the membrane-bound polyribosomes, since they constitute more than 70% of the total ribosomes in normal and regenerating liver (Zweig & Grisham, 1971), and the rates of synthesis *in vitro* are similar and even higher than the ones obtained in the absence of membrane. It is likely that the ribonucleases present in the endoplasmic-reticulum membranes, and relatively absent from the ribosomes, considerably affect the protein-synthesizing ability of the membrane-bound ribosomes. Therefore they are probably more active *in vivo* than the free ribosomes, an assumption that cannot be tested by merely comparing the rates of incorporation of [^{14}C]leucine *in vivo* owing to the continuous transfer of recently synthesized proteins to other parts of the cell. A conservative estimate of the proportion of cytochrome *c* synthesized by both ribosomal populations would then indicate that in the liver at least two-thirds of this protein is made by the rough endoplasmic reticulum.

This conclusion does not agree with the view that free polyribosomes are the exclusive sites of synthesis of proteins for the internal needs of the cell, as opposed to bound polyribosomes making only proteins for export. The only distinction so far found between the two ribosome classes resides in their mRNA (Murthy, 1972*b*; Gilbert, 1973) and membrane binding. However, there is considerable evidence ruling out the possibility that free ribosomes arise as artifacts of cell fractionation and that ribosomes are really all membrane-bound in the intact cell (see Campbell, 1970; Tata, 1971). Considering these facts, the hypothesis may be modified as follows. (*a*) The strict functional differentiation between free and membrane-bound polyribosomes applies only to the mechanism of transport involved, irrespective of the kind of protein synthesized. The ribosomes of the rough endoplasmic reticulum play a role whenever the protein has to be transported within the cisternae or tightly associated with a membrane flow, either to the outside of the cell (exportable proteins), or to internal membranous structures (membrane proteins; see Dallner *et al.*, 1966; Andrews & Tata, 1971). The free ribosomes synthesize those internal proteins that are entirely free in the cell sap or only loosely associated with membranes and that do not require a compartmentalized mechanism of transport. (*b*) When the endoplasmic reticulum is absent or considerably decreased (culture cells, tumours, non-secretory tissues etc), or when a protein normally exportable remains within the cell, free polyribosomes take the role normally reserved for the membrane-bound ribosomes, with the subsequent transport of the synthesized product through the cytosol. (*c*) In certain instances the same protein can be made simultaneously by both kinds of polyribosomes, assuring two ways of transport according to its ultimate destination, either compartmentalized in an organelle or free in the cytoplasm, as for catalase (Higashi *et al.*, 1972; Kashiwagi *et al.*, 1971). The existence of a soluble pool of a protein normally integrated into a cell structure would allow a flow of recently synthesized protein directly to the structure. The partial turnover of some membrane constituents would not require the simultaneous transport of multiple proteins, and hence the intervention of the endoplasmic-reticulum membranes would become unnecessary, since the soluble pools provide the source for the exchange of individual enzymes. This agrees

with the hypothesis that the enzymic composition of the membrane is in a state of flux and that enzymes can be added to and deleted from the pre-existing membrane (Arias *et al.*, 1969; Kuriyama *et al.*, 1969).

The mitochondria in liver are usually closely surrounded by the rough endoplasmic reticulum, and the outer membrane has been shown to have a composition resembling that of the smooth endoplasmic reticulum. It is therefore quite plausible that in secretory tissues, with a well-developed endoplasmic reticulum, mitochondrial proteins are mainly synthesized by membrane-bound ribosomes, thus allowing an efficient co-ordinate transport of enzymes and lipoproteins to the site of assembly. Certain soluble proteins, such as cytochrome *c*, would also be synthesized to a small extent by free ribosomes to facilitate some direct renewal of parts of an organelle without affecting the whole structure.

Partial hepatectomies were efficiently performed by Miss Emilia Pérez Ayuso, and the rats were provided by the Instituto de Medicina Experimental (Universidad Central de Venezuela), to whom our thanks are given. This work was supported by grant DF-S1-63 from the Consejo Nacional de Investigaciones Cientîficas y Tecnológicas (CONICIT). C. S. de C. is a member of the teaching staff of the Department of Physiological Sciences, Faculty of Medicine, Universidad Central de Venezuela.

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