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The Cell-Free Synthesis of Cytochrome *c* by a Microsomal Fraction from Rat Liver

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Conditions were investigated for demonstrating the synthesis *in vitro* of the complete molecule of cytochrome *c* by isolated liver microsomal systems from partially hepatectomized rats. It was first found that *in vivo* the early labelled cytochrome *c* associated with the microsomal fraction required, by comparison with the mitochondrial pool, more drastic conditions of extraction and its binding was less affected by freezing and thawing of the subcellular particles. The procedure of extraction and purification of cytochrome *c* had to be modified accordingly, to assure the recovery of the recently synthesized molecule. Several subcellular fractions were isolated from regenerating liver with a homogenization medium containing either 5 or 10mM-Mg^{2+} and most of them were active in the synthesis of the cytochrome *c* apoprotein. The microsomal fraction, in the presence of either cell sap or pH 5.0 fraction, was also able to incorporate [^{59}Fe]haemin, δ-amino[^{3}H]laevulic acid and ^{55}Fe into the prosthetic group of cytochrome *c*. These experiments confirm firmly the conclusions of our previous results obtained *in vivo* showing that both the apoprotein and the haem moieties are made and linked together on cytoplasmic ribosomes and only then is the complete molecule transferred to the mitochondria.

By studying the kinetics of the incorporation *in vivo* of [^{14}C]lysine into the cytochrome *c* and total protein of different subcellular fractions of rat liver, it was shown that cytochrome *c* is synthesized *in toto* by the microsomal ribosomes and later transferred to the mitochondria (González-Cadavid & Campbell, 1967*b*,*c*; González-Cadavid, Bravo & Campbell, 1968). These results confirmed earlier suggestions based on more indirect evidence (Roodyn, Suttie & Work, 1962; Haldar, Freeman & Work, 1966; Beattie, Basford & Koritz, 1966), and were later supported by similar studies of Kadenbach & Urban (1968), and Kadenbach (1968, 1969) in rat liver *in vivo*. By substituting δ-amino-[^{3}H]laevulic acid for [^{14}C]lysine as the radioactive precursor, Penniall & Davidian (1968) and Davidian, Penniall & Elliot (1969) also substantiated that the complete assembly of the protein occurs in the endoplasmic reticulum. Cytochrome *c* was the first specific mitochondrial protein whose synthesis was shown to take place outside the organelle, thus reinforcing the hypothesis of a restricted mitochondrial autonomy (Roodyn & Wilkie, 1968; Work, 1970).

However, Kadenbach (1970) modified his earlier conclusions, on the grounds that when [^{14}C]leucine was given *in vivo* to rats, the chromatographic peak of radioactive protein (identified as the apoprotein) appeared just before the peak of microsomal cytochrome *c*. Also, the injection of ^{59}Fe labelled both the mitochondrial and the microsomal cytochrome *c* at the same rate, showing that the latter cannot be a precursor of the former. These results were interpreted as indicating that the microsomal fraction can only synthesize the apoprotein moiety, whereas the linkage to the haem takes place after the transfer of the protein chain to the mitochondria. Kadenbach (1970) assumed that his previous results (Kadenbach, 1968) were due to an inefficient purification of the microsomal cytochrome *c*.

Although several experiments could be devised to decide between the two alternatives, the best answer would come from the complete synthesis of the haemoprotein by an isolated microsomal fraction. Our previous attempts with rat liver microsomal fraction under conditions that allowed the energy-dependent incorporation of amino acids into total protein and serum albumin were unsuccessful (González-Cadavid & Campbell, 1967*a*), and we attributed the failure to the inability of the apoprotein to conjugate with the ferriporphyrin

ring. However, other explanations were equally possible, such as that the newly synthesized cytochrome *c* could be left unextracted or be lost during the purification process (González-Cadavid & Campbell, 1967*b*), or that the mRNA for the haemoprotein is particularly unstable. In the present work we tried to avoid these difficulties by searching for the optimum incubation conditions and applying a sequential procedure for the extraction of the more tightly bound cytochrome *c*. It is shown that the isolated microsomal fraction from regenerating rat liver is able to incorporate [^{14}C]-leucine, [^{59}Fe]haemin, δ-amino[^{3}H]laevulic acid, and ^{55}Fe, into purified cytochrome *c*, thus clearly demonstrating that the synthesis of the complete molecule takes place in the cytoplasmic ribosomes. So far as we are aware, this is the first report on the synthesis of a specific mitochondrial protein by a cell-free system.

MATERIALS AND METHODS

Chemicals. L-[U-^{14}C]Leucine (sp. radioactivity 344 mCi/mmol), [^{59}Fe]ferric chloride (sp. radioactivity 10 Ci/g of Fe), L-[U-^{14}C]lysine (sp. radioactivity 312 mCi/mmol) and [^{55}Fe]ferric chloride (sp. radioactivity 8 Ci/g of Fe) were obtained from Amersham–Searle Corp., Des Plaines, Ill., U.S.A. δ-Amino[2,3-^{3}H]laevulic acid hydrochloride (sp. radioactivity 7 Ci/mmol) was purchased from Schwarz BioResearch Inc., Orangeburg, N.Y., U.S.A.). The [^{14}C]-leucine was diluted to the desired concentration and made iso-osmotic with 0.77 M-NaCl. The [^{14}C]lysine solution was freeze-dried and dissolved for injection in 0.2 M-sodium phosphate buffer, pH 7.4. The [^{59}Fe]ferric chloride and [^{55}Fe]ferric chloride were evaporated to dryness by heating in a water bath at 60°C under N_2, and addition twice of a few drops of water to assure the complete elimination of HCl. The [^{59}Fe]ferric chloride was then treated as described below. The [^{55}Fe]ferric chloride was dissolved in 0.15 M-NaCl. The δ-amino[^{3}H]laevulic acid was diluted to the desired concentration and made 20 mM with respect to tris by the addition of 0.5 M-tris–HCl, pH 7.4.

The following biochemical compounds were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A.: ATP as the disodium salt (grade I); Triton X-100; cytochrome *c* (type III, from horse heart); dithioerythritol; phosphoenolpyruvate (K^+ salt); pyruvate kinase (type II); tris as 'Trizma base'; CM-cellulose (0.7 mequiv./g); DEAE-cellulose (0.9 mequiv./g); Sephadex G-25 (80 μm bead size); haemin (type I; bovine); GTP as the sodium salt (type III). The L-amino acids (chromatographic grade) and the haematoporphyrin dihydrochloride were purchased from Mann Research Laboratories, New York, N.Y., U.S.A. Amberlite IRC-50 was obtained as the CG-50 A.R. chromatographic grade (200–400 mesh) from Mallinckrodt Chemical Works, St Louis, Mo., U.S.A. Protoporphyrin (disodium salt) was from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.

2,5-Diphenyloxazole and 1,4-bis-(5-phenyloxazol-2-yl) benzene, both scintillation grade, were purchased from Nuclear–Chicago Corp., Des Plaines, Ill., U.S.A. The phosphoenolpyruvate (barium–silver salt) replacing occasionally the Sigma product, was from Boehringer und Sohne G.m.b.H., Mannheim, Germany, and was transformed into the potassium salt with HCl and K_2SO_4. All other chemicals were of analytical grade whenever commercially available. The glass-fibre filter discs (2.4 cm) were Whatman grade GF/A and purchased from H. Reeve Angel and Co. Ltd., London, U.K., or the equivalent grade 934 AH from Reeve Angel, Clifton, N.J., U.S.A. The dialysis tubing was Visking of 28 mm inflated diameter (from Gallenkamp and Co. Ltd., London, U.K.), and was boiled for 30 min in 1% $NaHCO_3$ before use.

The ATP, phosphoenolpyruvate and GTP were mixed at tenfold the appropriate concentrations for final use in a single solution in twice-distilled water designated '10× energy mixture' adjusted to pH 7.4 (against glass electrode) with 1 M-KOH at 0°C, and stored at −20°C in several vials. This solution contained 20 mM-ATP, 100 mM-phosphoenolpyruvate, and 2.5 mM-GTP. The phosphokinase [10 mg/ml in $(NH_4)_2SO_4$] was diluted with twice-distilled water to 1 mg/ml, and stored in the same way as the preceding solution.

The Amberlite IRC-50 and the Sephadex G-25 were washed and equilibrated as described by González-Cadavid & Campbell (1967*b*).

Equipment. The tissue press, made to the design of Porterfield (1960), was purchased from Climpex Ltd., London N.W.7, U.K. All the spectra were recorded in a Beckman model DB-G double-beam spectrophotometer. The sonications were carried out with an MSE 100 W ultrasonic disintegrator. The refrigerated centrifuges used were the Spinco L2-65 B (Beckman) and the Sorvall RC-2-B. The radioactivities of the samples were counted in an automated liquid-scintillation spectrometer (model 720, Nuclear–Chicago Corp.).

Animals. Sprague–Dawley rats from a closed colony bred at the Instituto Venezolano de Investigaciones Científicas, Caracas, Venezuela, were used throughout. Partial hepatectomy was performed by the technique described by Higgins & Anderson (1931) on male rats (body wt. 150±10 g), and liver regeneration was allowed to proceed for 48 h. The rats were maintained with a 10% (w/v) glucose solution for the first 24 h and food was restored 4 h after the operation and withdrawn 18 h before the rats were killed. For the preparation of 'carrier' mitochondria, rats of both sexes (body wt. 250–300 g) were used and starved overnight. The rats were stunned with a blow on the head and killed by decapitation.

Preparation of a 'carrier' source of cytochrome c. About 100 g of liver was rinsed several times in ice-chilled 0.3 M-sucrose and the mitochondria were obtained by the procedure described by González-Cadavid & Campbell (1967*b*). The larger amounts of tissue homogenates were processed by using the GSA rotor of the Sorvall Superspeed RC-2 centrifuge instead of the SS-34 rotor only in the first 600*g* centrifugation. The mitochondrial pellets were homogenized in 50 ml of water, protein was determined as described below, and the concentration was adjusted to 40 mg of protein/ml. The suspension was divided in 7 ml portions which were kept at −20°C until use.

Subcellular fractionation. In the experiments *in vivo*, the subcellular fractions from normal rat liver were obtained exactly as described by González-Cadavid &

Campbell (1967*b*) and kept either frozen at −20°C or in ice until analysis.

The preparation of the fractions from regenerating liver used for the cell-free incubations was done by two different procedures.

Procedure A. The livers from four partially hepatectomized rats were weighed and rinsed several times with ice-chilled 0.25M-sucrose. The livers were then forced through a tissue press. All operations thereafter were performed at 2–4°C. The mince from each liver was suspended in 12ml of medium *A*, containing 5mM-$MgCl_2$, 50mM-tris buffer, pH7.8 at 20°C, 25mM-KCl and 0.25M-sucrose (Rendi & Hultin, 1960), and separately homogenized in a Potter–Elvehjem homogenizer with a Teflon pestle (diam. 19mm) with 0.10–0.15mm clearance from the glass mortar. Three strokes were used over a total period of 90s with a constant rheostat setting such that the pestle rotated at about 800 rev./min. The homogenates were pooled and made up to 90ml with medium *A* and centrifuged (600*g*) for 10min. The supernatant was decanted off and the pellet was discarded. A portion (one-quarter of the total volume) was centrifuged at 105000*g* for 60min. The pellet was designated the 'mitochondrial–microsomal fraction' and the supernatant the 'cell sap'. The 'pH5.0 fraction' was prepared from the cell sap as indicated by von der Decken & Campbell (1962). Another portion (one-quarter of the total volume) was kept as such and designated the 'post-nuclear fraction'. The remaining volume was centrifuged at 15000*g* for 10min. The supernatant was decanted off leaving only the firm portion of the sediment, which was discarded. The supernatant was designated the 'post-mitochondrial fraction'. In one experiment the 15000*g* centrifugation for 10min stage was performed at 105000*g* for 10min and the corresponding supernatant was designated 'light microsomal–cell sap fraction'. All fractions were kept in ice until use.

Procedure B. Only three livers were used and the homogenization was done as described above but in 2 vol. of medium *B* containing 10mM-$MgCl_2$, 35mM-tris buffer, pH7.8 at 20°C, 25mM-KCl and 0.15M-sucrose (Rendi & Hultin, 1960). The nuclei and mitochondria were eliminated at 12000*g* for 10min. The supernatant was decanted by inverting the tube leaving only the firm portion of the sediment, which was designated the '10mM-Mg^{2+}-post-mitochondrial fraction'. The remaining volume was centrifuged at 105000*g* for 60min. Most of the supernatant was removed by suction, the upper section contaminated by lipids being discarded, and the clear portion was designated 'cell sap'. The rest of the supernatant was decanted off, leaving only the firm portion of the sediment, which was designated 'microsomal fraction'. A portion (2.5ml) of the cell sap was used for the preparation of the 'pH5 fraction' as indicated above.

Preparation of [^{59}Fe]*haemin*. A modification of the procedure described by Falk (1964) was used for the insertion of the ^{59}Fe into the porphyrin ring. To 200μCi of [^{59}Fe]ferric chloride, evaporated to dryness as described under 'Chemicals', 5ml of acetic acid containing 0.1ml of 0.05M-$FeSO_4$, freshly prepared and previously gassed with N_2, was added. This solution was transferred to a 25ml conical flask stoppered with a gas-washbottle head and then heated in a water bath to 80°C, under a N_2 closed atmosphere. A portion (0.1ml) of a 10mg/ml solution of haematoporphyrin (1.69μmol) was immediately added and the heating proceeded for 10min. Autoxidation was then allowed to occur for 20min at room temperature and the solution was evaporated to dryness under a current of N_2, at 80°C. The residue was suspended in 5ml of a 0.2M-$FeCl_3$ solution in 0.01M-HCl and retained on a fritted glass filter, where it was washed with several 5ml portions of the same solution. Water was then passed through until the yellow colour was removed, and 10ml of 25% (v/v) HCl was used for extracting on the filter the residual porphyrin. The haemin was dissolved by passing several 0.5ml portions of 0.01M-NaOH and 0.5ml of 0.5M-tris–HCl buffer, pH7.8 was added. The pH was adjusted to 7.8 with 1M-HCl, and the volume made up to 5.0ml with water. A 0.05ml portion was used for recording the spectrum of the pyridine haemochromogen (Morrison & Horie, 1965) in a total volume of 1.5ml, and from this dilution a new 0.05ml portion was taken for radioactivity counting as described below.

With a Fe^{2+}/haematoporphyrin molar ratio of 3:1 the recovery was 40% in terms of the porphyrin input and the total radioactivity obtained was 36μCi, or 13% of the [^{59}Fe]ferric chloride input, which agreed well with the 3/1 ratio. Other molar ratios were tried (12/1; 24/1) but the haemin yield was not significantly increased and the ^{59}Fe recovery was considerably lowered, owing to the dilution of the original radioactivity. Although this low yield differed from the 95% conversion reported by Falk (1964) for non-radioactive $FeSO_4$, it was acceptable in view of the restraints imposed by the labelling reaction itself.

Incubation conditions. The incubation of the cell-free systems was performed as indicated in the respective experiments.

Extraction and purification of cytochrome c. Different procedures were applied according to the origin of the samples. All the operations described were done at 1–4°C. For the subcellular fractions obtained in the experiments *in vivo*, shown in Tables 1 and 2, the aims were to investigate the relations between the degree of extractability of cytochrome *c* and the sequence of synthesis of each pool. The method employed was designated no. 1 and was as follows. The mitochondrial and microsomal fractions were homogenized in 20ml of 0.15M-NaCl, left for 30min and then centrifuged at 145000*g* for 90min. The clear supernatants were kept in an ice bath and the pellets were re-extracted sequentially under identical conditions with the same volume of water, 0.15M-NaCl, and 0.15M-NaCl, followed by sonication in 2ml portions (1min each) at 8.5μm of amplitude. The NaCl extracts were diluted 1:6 and all supernatants were adjusted to pH4.0 with 0.25M-H_2SO_4, left for 30min with occasional stirring and then centrifuged at 15000*g* for 30min. The pellets were kept and the supernatants were adjusted to pH6.5 with 0.5% NH_3. Some of the suspensions became turbid and were clarified by centrifugation at 15000*g* for 10min. All sediments obtained from the different extracts of each subcellular fraction were pooled with the residue of the last respective extraction, and were re-homogenized in water. The pH was adjusted to 4.0, and the supernatant was adjusted to pH6.5 as described above. The extraction was repeated with 0.15M-NaCl under the same conditions. The pellets were finally homogenized in 0.1M-ammonium acetate,

adjusted to pH7.0 with 5% (w/v) NH_3, and 15% (w/v) sodium deoxycholate was added, to a final concentration of 1.5%. After centrifugation at 15000*g* for 30min, the supernatant was dialysed against five changes of 0.1M-ammonium phosphate (pH8.2)–0.15M-NaCl, and three changes of 0.15M-NaCl. The seven different extracts from each subcellular fraction were passed through Amberlite columns and purified exactly as described by González-Cadavid & Campbell (1967*b*,*c*).

The main problem arising with the incubation of the cell-free systems was how to extract even the smallest cytochrome *c* pool, because the recently synthesized protein could be precisely the more resistant to liberation from the subcellular particle. When the incubations were performed with radioactive amino acids, the method used (no. 2) was as follows. A suitable portion of the suspensions (about 2% of the total volume) was separated for the measurement of the total protein radioactivity and the mitochondrial carrier source of cytochrome *c* was added to all mitochondrial-free samples. The suspensions were adjusted to pH4.0 with 5.0M-H_2SO_4 and left for 60min with occasional stirring and, if necessary, pH adjustment. After centrifugation at 30000*g* for 15min, the supernatant was kept and the pellet was homogenized in 10ml of 1M-NaCl and ultrasonicated as described above. A new centrifugation was performed at 30000*g* for 15min and the supernatant was pooled with the pH4.0 extract. The pellet was re-extracted twice with 10ml of 0.15M-NaCl, and in the second treatment the pH was adjusted to 9.0 with 5% NH_3 and the mixture left for 30min. The supernatant obtained by centrifugation at 30000*g* for 15min was pooled with the previous extracts, adjusted to pH7.0 and dialysed against tap water followed by several changes of 0.02M-ammonium acetate. The contents of the dialysis tubing were adjusted to pH4.0 and centrifuged for 10min. The extract was neutralized, oxidized with $K_3Fe(CN)_6$ (0.5mM final concentration) and passed through a single column (1cm diam.×5cm) of Amberlite CG-50, equilibrated at pH7.0. Thereafter the procedure of purification was the same as described by González-Cadavid, Wecksler & Bravo (1970).

Method no. 3 was applied to the cell-free systems incubated with radioactive precursors of the haem moiety of cytochrome *c*. In this case, the complete purification of the protein was considered unnecessary, since the precursors would only be incorporated into the prosthetic group of the haemoproteins (see Aschenbrenner, Druyan, Albin & Rabinowitz, 1970, and the references listed there). It was therefore possible to omit several chromatographic steps, provided all other haemoproteins were completely eliminated, as judged from the spectrum of the fraction containing cytochrome *c*. This was achieved by introducing a treatment with ammonium sulphate before dialysis. At the same time the procedure was slightly modified to obtain an even more thorough extraction of cytochrome *c*. The details are as follows.

The post-mitochondrial and microsomal suspensions were treated as described under method no. 2 up to the ultrasonication. The residue remaining from this extraction was homogenized in 5ml of 1% $Al_2(SO_4)_3$ and the pH adjusted to 4.0. After 1h, the suspension was centrifuged at 30000*g* for 10min and the supernatant was kept. The sediment was suspended in 5ml of 0.25M-$(NH_4)_2HPO_4$ and adjusted to pH8.0 with 5% NH_3. All the supernatants were pooled, neutralized with 5% NH_3 and made 80% saturated with powdered $(NH_4)_2SO_4$. The precipitate was removed by centrifugation at 30000*g* for 15min and then dialysed as described in method no. 2. No precipitation occurred inside the dialysis tubing and the oxidized solution was directly adsorbed in the same Amberlite column as described above (except that it was in the Na^+ form). The washing was done with 30ml of 0.1M-sodium phosphate buffer, pH7.0, containing 0.5mM-$K_3Fe(CN)_6$. After eliminating the $K_3Fe(CN)_6$ with water, the cytochrome *c* was eluted with 0.5M-sodium phosphate buffer, pH7.0. At this point, asi n each of the extraction steps, the spectrum was recorded both in the oxidized and in the reduced state.

Finally, a simplified version of method no. 3 was applied when the addition of the mitochondrial carrier source of cytochrome *c* was omitted. In this case (method no. 4), the incubated post-mitochondrial and microsomal suspensions were diluted 1:4 with water, adjusted to pH4.0 and after 30min centrifuged at 30000*g* for 15min. The supernatant was adjusted to pH6.5, centrifuged if necessary, and oxidized with $K_3Fe(CN)_6$ (0.5mM final concentration). It was then passed through a column (0.6cm diam.×2cm) of Amberlite CG-50, equilibrated at pH7.0. The residue from the pH4.0 extraction was suspended in 5ml of 0.15M-NaCl and after 30min the homogenate was centrifuged at 30000*g* for 15min. The supernatant was diluted 1:5, neutralized, oxidized and finally passed through the same Amberlite column as described above. Cytochrome *c* was washed, eluted and determined as indicated in method no. 3.

Preparation of samples for the determination of radioactivity. Portions (0.1ml) were taken from the samples of the subcellular suspensions previously separated for the determination of total protein radioactivity. They were adsorbed directly or after suitable dilution, on to glass-fibre discs (24mm diam.) and processed by a modification of the method of Mans & Novelli (1961) including successive washings with 10% (w/v) trichloroacetic acid (containing 1mg of L-leucine, L-lysine or ferric chloride/ml, according to the precursor used), 5% (w/v) trichloroacetic acid (once at 90°C for 15min, and twice at room temperature), ethanol–ether (3:1, v/v) (three times), acetone and ether (twice). When haem precursors were used as radioactive tracers, the process included three washings with acetone–0.01M-HCl before the ether to remove any non-covalently linked haem. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

The cytochrome *c* samples from the experiments *in vivo*, isolated and purified by method no. 1, were prepared for radioactivity counting as described by González-Cadavid & Campbell (1967*c*). The cytochrome *c* samples from the incubations with ^{14}C-labelled amino acids (method 2) were prepared for radioactivity counting as indicated by González-Cadavid *et al.* (1970). Finally, in the incubation with the radioactive precursors of the prosthetic group (methods 3 and 4), cytochrome *c* was precipitated directly from the eluates by the addition of trichloroacetic acid (20% final concentration), in the presence of 0.1% *o*-phenanthroline to chelate any free iron which could induce the formation of a precipitate of $Fe_4[Fe(CN)_6]_3$, and 0.5mg of horse heart cytochrome *c* as 'carrier'. The precipitate was collected on to the glass-

fibre discs by filtration and washed three times with 10% trichloroacetic acid, three times with acid–acetone, once with 30% (v/v) H_2O_2 to bleach the sample, and then with ether.

Radioactivity measurements. All the total protein samples on 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. The cytochrome *c* samples dissolved in 0.2–0.4ml of formic acid were mixed with 10ml of the scintillation fluid tT21 proposed by Patterson & Greene (1965), containing 2 vol. 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. Precautions were taken to avoid the sedimentation of cytochrome *c* during a prolonged period of counting, by shaking each vial immediately before its positioning in the counting station. The cytochrome *c* samples adsorbed on the glass-fibre discs were counted for radioactivity in vials with 2ml of toluene containing 0.9% of 2,5-diphenyloxazole and 0.1% of 1,4-bis-(5-phenyloxazol-2-yl)benzene. All the vials were counted in an automatic liquid-scintillation spectrometer to a 1.5% statistical error at 68% probability.

RESULTS

Metabolic heterogeneity of cytochrome c. One of the main problems to be considered in any attempt to synthesize cytochrome *c* by a cell-free system is the possibility that the protein could be left unextracted or be lost during the isolation and purification process. The straightforward application of a single procedure to the different subcellular fractions, incubated without proper attention being paid to this possible source of error, could lead to erroneous results according to the organelle extracted and the tightness of binding of the cytochrome *c* pools. We had some indication in this sense from previous work (González-Cadavid & Campbell, 1967*b*,*c*; González-Cadavid *et al.* 1968) where we showed the existence of two metabolically distinct pools, extractable from the stored-frozen fractions with water and 0.15M-sodium chloride, whose proportion differed in each subcellular fraction.

We now wanted to investigate whether this was a simplified version of a more complex situation *in vivo* characterized by a great number of pools, and whether the newly synthesized protein was in fact the most firmly linked to the original organelle structure, i.e. the fraction not submitted to freezing and thawing. Two experiments were performed where groups of rats were given a pulse of [^{14}C]-lysine *in vivo* and the cytochrome *c* was isolated from either the fresh or the stored-frozen subcellular fractions, under a sequence of different treatments. Table 1 shows that in the mitochondrial fraction only 14% of the total cytochrome *c* could be isolated from the fresh organelle by 0.15M-sodium chloride and water in this order. However, when the mitochondrial structure was disrupted by freezing and thawing, this amount rose to 74%. With the fresh fraction, osmotic shock rendered extractable by 0.15M-sodium chloride a further 62%, whereas only 11% was obtained from the

Table 1. *Amounts of cytochrome* c *extracted from the mitochondrial and microsomal fractions of rat liver under a sequence of different treatments*

In each experiment six rats were injected in the sublingual vein with 15μCi of [^{14}C]lysine dissolved in 0.25ml of 0.2M-sodium phosphate buffer, pH7.4, and killed after 15min. Liver subcellular fractionation was done as described in the text (procedure A), and cytochrome *c* was extracted in several steps, either from the fresh (expt. no. 1) or from the stored-frozen samples (expt. no. 2), followed by purification as indicated in the text (method no. 1).

	Cytochrome *c* extracted (% of sum of extracts) from				
	Mitochondrial fraction		'Heavy' microsomal fraction	'Standard' microsomal fraction	
Sequence of extractions	Fresh	Stored-frozen	Fresh	Fresh	Stored-frozen
A at pH7					
1. 0.15M-NaCl	8.2	74.0	9.6	29.7	36.9
2. Water	5.8	0.4	4.6	2.5	4.2
3. 0.15M-NaCl	61.9	10.6	55.1	19.7	21.8
4. 0.15M-NaCl and ultrasonication	18.3	12.0	22.5	25.7	22.1
B at pH4					
5. Water	1.8	2.2	4.1	13.4	11.3
6. 0.15M-NaCl	0.6	0.6	2.0	5.2	3.8
C at pH7					
7. 1.5% Sodium deoxycholate	3.6	—	2.1	3.8	—
Sum of extracts (μg/g of liver)	35.5	56.5	38.3	10.2	13.4

stored-frozen organelle. In both cases about 12–20% of the cytochrome *c* was still associated with the mitochondrial structure, and most of it was extracted by 0.15M-sodium chloride and ultrasonication. A minor portion (3–5%) could only be obtained at pH 4.0 or with deoxycholate.

The situation with the standard microsomal fraction (6300000*g*-min) was different, since the disruption by freezing and thawing did not markedly change the cytochrome *c* distribution. Some 32–40% was extractable by 0.15M-sodium chloride followed by water, and only about 20% remained extractable by ultrasonication, and the pool requiring more drastic conditions for extraction (pH 4.0, deoxycholate) was quite considerable: 23% (fresh) or 15% (stored-frozen). The heavy-microsomal fraction (sedimenting between 80000 and 200000*g*-min), characterized by the presence of a high proportion of mitochondria, showed features of cytochrome *c* extraction resembling more the mitochondrial than the 'standard' microsomal fraction.

Table 2 shows that from a metabolic point of view, there was a certain heterogeneity in the mitochondrial pools, the more highly labelled cytochrome *c* (extracted with water at pH 4.0) having a specific radioactivity about twofold that of the less radioactive fraction that was the most abundant. In the 'standard' microsomal fraction the metabolic heterogeneity was much more evident, and the newly synthesized cytochrome *c* was very resistant to isolation, since it required the lowering of the pH to 4.0 for isolation. The specific radioactivity of this pool was particularly high when the microsomes had been frozen and thawed, probably due to the fact that the cytochrome *c* removed by the previous extractions (when compared with the fresh particles), was poorly labelled. The specific radioactivity of these two pools extracted at pH 4.0 was about 12-fold and 15-fold higher than those of the less radioactive microsomal pool and of the average mitochondrial cytochrome *c* respectively. The situation with the 'heavy' microsomal pools was again the product of the cross-contamination of mitochondria and the microsomal fraction, with preponderance of the former.

These experiments proved conclusively that the metabolic heterogeneity of cytochrome *c* was of particular importance for the microsomal fraction, and that the newly synthesized protein could escape extraction, therefore detection, if relatively drastic conditions of isolation were not used, even when more than 80% of the microsomal cytochrome *c* could have been obtained.

Incorporation of radioactive leucine into cytochrome c *by cell-free systems.* Our approach was to determine first whether the disruption of the hepatocyte plasma membrane stopped completely the synthesis of cytochrome *c*. Preliminary experiments showed that liver homogenates carry out a residual synthesis of cytochrome *c* and the ratio of its specific radioactivity to that of total protein is similar to or even higher than that obtained when the cell structure is preserved in the incubation of liver slices. It was decided therefore to try different post-nuclear systems lacking one or the other of the subcellular constituents possibly involved in the synthesis of cytochrome *c*. Since the experiments described in the preceding section demonstrated the very tight binding of the newly synthes-

Table 2. *Metabolic heterogeneity of mitochondrial and microsomal cytochrome* c *labelled* in vivo *as related to its sequence of extraction*

For experimental details see legend to Table 1.

	Sp. radioactivity of cytochrome *c* (d.p.m./mg)				
	Mitochondrial fraction		'Heavy' microsomal fraction	'Standard' microsomal fraction	
Sequence of extractions	Fresh	Stored-frozen	Fresh	Fresh	Stored-frozen
A at pH 7					
1. 0.15M-NaCl	740	556	604	1072	748
2. Water	636	—	704	—	—
3. 0.15M-NaCl	540	640	432	980	1456
4. 0.15M-NaCl and ultrasonication	684	716	2064	1916	2940
B at pH 4					
5. Water	1248	1108	1180	3852	9396
6. 0.15M-NaCl	—	852	—	4560	8188
C at pH 7					
7. 1.5% Sodium deoxycholate	892	—	—	—	—
Sum of extracts	612	600	—	1828	2576

ized cytochrome c to the microsomal particles, care was taken to apply a sequence of extraction in various steps (method 2) to assure the complete removal of the protein from the incubated subcellular fractions. The selection of regenerating liver was based on the active mitochondrial biogenesis occurring after partial hepatectomy.

Another point that was considered in the present experiments was the possibility that the microsomal preparations could require exogenous haemin for the synthesis of the complete molecule, and thus when the systems were deprived of mitochondria, haemin was added to the incubation. Since the linkage between the cytochrome c apoprotein and its prosthetic group is by a thioether bridge, a thiol-group-protecting reagent (dithioerythritol) was sometimes used. Finally, *N*-acetylglycine, being the *N*-terminus of the chain and the initiation point for the synthesis, was thought to be a possible limiting factor in the activity of the systems, and was also added in certain cases. The results presented in Table 3 for the subcellular fractions isolated in a 5mM-Mg^{2+}-containing medium show that in most samples from two different experiments there was a significant incorporation of [^{14}C]leucine into cytochrome c, and that the most active system was the 'post-mitochondrial fraction' (microsomal fraction+cell sap) in the presence of *N*-acetylglycine and dithioerythritol. The elimination of part of the bound ribosomes and the heavier polyribosomes by differential centrifugation yielding the 'light microsomal fraction+cell sap', decreased the incorporation. The ratio of the radioactivity incorporated into cytochrome c to that present in total protein was in the best case, 0.08%, which was less than 70% of the ratio obtained with the slices. This indicates that the synthesis of cytochrome c proceeded for a shorter period than that of total protein. Practically no incorporation occurred with the mitochondrial–microsomal fraction in the presence of the pH 5.0 fraction, probably owing to the inhibition of the microsomal ribosomes by lysosomal ribonuclease not counteracted by the ribonuclease inhibitor of the cell sap (Bloemendal, Bont & Benedetti, 1964).

We assumed that the mRNA for cytochrome c was particularly unstable and that perhaps the Mg^{2+} concentration employed for the isolation of

Table 3. *Incorporation of* [^{14}C]*leucine into cytochrome* c *by cell-free systems from regenerating liver isolated with a* 5mM-Mg^{2+}*-medium*

The subcellular fractions were isolated from four regenerating livers as described in the text (procedure A), by using a homogenization medium containing $MgCl_2$ (5mM), tris buffer (50mM) (pH 7.8 at 20°C), KCl (25mM) and sucrose (0.25M). The fractions from one liver each were incubated for 30min at 37°C in a medium containing sucrose (90mM), Mg^{2+} (6mM), K^+ (15mM), tris (21mM), phosphoenolpyruvate (10mM), GTP (0.25mM), ATP (2mM), pyruvate kinase (50μg/ml), [^{14}C]leucine (1.33μCi/ml, except in 1 and 5 with 4μCi/ml), cytochrome c (3.3μg/ml), and when indicated, haemin (8μM), acetylglycine (2.5mM) and Cleland's reagent (1.0mM), all in a total volume of 30ml (except in 1 and 5, with 10ml). Total protein and cytochrome c were prepared for radioactivity determinations as described in the text (method no. 2).

Incubated fractions	Addition of acetylglycine and Cleland's reagent	Protein specific radioactivity referred to equivalent microsomal protein* (d.p.m./mg)	Total radioactivity in cytochrome c: Measured (d.p.m.)	Corrected for recovery (d.p.m.)	Ratio to that in total protein (%)
Experiment 1					
1. 'Mitochondrial–microsomal'+ 'pH 5 fraction'	—	6380	6	35	0.039
2. 'Post-nuclear'	—	6150	114	407	0.065
3. 'Post-mitochondrial' (+haemin)	—	9470	75	326	0.068
4. 'Post-mitochondrial' (+haemin)	+	—	251	1093	—
Experiment 2					
5. 'Mitochondrial-microsomal'+ 'pH 5 fraction'	—	18100	0	0	0
6. 'Post-nuclear'	—	—	39	195	—
7. 'Light microsomal and cell sap' (+ haemin)	—	13320	14	70	0.010
8. 'Light microsomal and cell sap' (+haemin)	+	10050	81	405	0.079

* Microsomal protein calculated to be present in the incubation.

Table 4. *Incorporation of* [^{14}C]*leucine into cytochrome* c *by cell-free systems from regenerating liver isolated with a* 10mM-Mg^{2+}*-medium*

The subcellular fractions were isolated from three regenerating livers as described in the text (procedure B), with a homogenization medium containing $MgCl_2$ (10mM), tris buffer (35mM) (pH 7.8 at 20°C), KCl (25mM) and sucrose (0.15M). The conditions of incubation were as indicated in Table 3, except for the final volumes that were 15ml in the post-mitochondrial fraction and 5ml in the microsomal fractions. The concentration of [^{14}C]leucine was 2μCi/ml in all cases and the microsomal protein present in the incubation was around 3mg/ml in the post-mitochondrial fraction and 8mg/ml in the microsomal fractions. All incubations contained haemin, acetylglycine and Cleland's reagent. Total protein and cytochrome *c* were prepared for radioactivity determinations as described in the text (method 2).

Incubated fractions	Protein sp. radioactivity referred to equivalent microsomal protein* (d.p.m./mg)	Total radioactivity in cytochrome *c*		
		Measured (d.p.m.)	Corrected for recovery (d.p.m.)	Ratio to that in total protein (%)
1. 'Post-mitochondrial'	67370	91	217	0.006
2. 'Microsomal'+cell sap	49620	76	220	0.011
3. 'Microsomal'+pH 5 fraction	35340	114	329	0.023

* Microsomal protein calculated to be present in the incubation.

the subcellular fractions (5mM) was not the optimum. Therefore in a new experiment the subcellular fractions were isolated in a medium containing 10mM-Mg^{2+} and only the most active of the systems previously tested, the post-mitochondrial fraction, was used. To assess the influence of the cell sap on the activity of the microsomal fraction, two more conventional systems were also tried. One containing only one-tenth of the cell sap present in the post-mitochondrial fraction, 'microsomal fraction+cell sap', and in the other the pH 5.0 fraction was substituted for the cell sap. Table 4 shows that the increase in Mg^{2+} concentration enhanced markedly the protein-synthesizing ability of the post-mitochondrial fraction, as expressed per mg of microsomal protein present in the incubation. The removal of most of the cell sap or its replacement by the pH 5.0 fraction decreased considerably the equivalent activity. The incorporation into cytochrome *c* was adequate in the three cases, but when compared with the efficiency of total protein synthesis the ratio was lower than with the fractions isolated with 5mM-Mg^{2+}. The highest ratio was found with the 'microsomal+pH 5.0 fraction'.

Incorporation of radioactive precursors into the prosthetic group of cytochrome c *by cell-free systems.* Having demonstrated the incorporation of labelled amino acids into the cytochrome *c* apoprotein, our next step was to investigate whether the synthesis of its prosthetic group takes place under our conditions *in vitro*. At this point we had to choose between 5 and 10mM-Mg^{2+} for the isolation of the subcellular fractions. We considered that the specific radioactivities of cytochrome *c* are not an absolute indication, owing to the possible unstability of its mRNA, whereas the synthesis of total proteins seemed to be much more reproducible. This process was clearly favoured by 10mM-Mg^{2+}, and since it allowed a reasonable labelling of cytochrome *c*, all further studies were conducted under these conditions of isolation. Table 5 shows that there was a significant incorporation of all the three precursors of the haem moiety, namely haemin, δ-aminolaevulic acid and iron. The labelling ratio to that of total protein was poor in comparison with the cell-free systems tested with [^{14}C]leucine, except for the 'microsomal+pH 5.0 fraction', which was precisely the most efficient system for the synthesis of the cytochrome *c* apoprotein.

The last experiment was devised to rule out any possible binding of a basic labelled haemoprotein to the cytochrome *c* that could arise from the large amount of acidic proteins present in the mitochondrial carrier source. The incubations with the 'microsomal+pH 5.0 fraction' were carried out with δ-amino[^{3}H]laevulic acid as described above, but before extraction one sample received no carrier whatsoever and the other only 1.0mg of horse heart cytochrome *c*. In both instances (see Table 5, samples 10 and 11) the incorporation into the purified cytochrome *c* was close to the ones obtained with the sample (no. 6) where a carrier mitochondrial source had been added.

DISCUSSION

The main point to be examined is whether the incorporation of radioactive precursors into cytochrome *c* obtained in our cell-free systems is really

Table 5. *Incorporation of radioactive precursors of haem into the prosthetic group of cytochrome* c *by cell-free systems from regenerating liver, obtained with a* 10mM-Mg^{2+}-*medium*

The subcellular fractions were isolated from three regenerating livers and the incubations performed essentially as described in Table 4, except for sample 10 where two livers were used and the incubation volumes were doubled accordingly. No unlabelled haemin was added. Total protein and cytochrome *c* were prepared for radioactivity determinations as described in the text under method 3 (samples 1–3; mitochondrial carrier source) or method 4 (sample 10, no carrier; sample 11, horse heart cytochrome *c* as carrier).

Incubated fractions	Total sp. radioactivity referred to equivalent microsomal protein* (d.p.m./mg)	Total radioactivity in cytochrome *c*		
		Measured (d.p.m.)	Corrected for recovery (d.p.m.)	Ratio to that in total protein (%)
Expt. 1. With 1 μCi of [^{59}Fe]haemin/ml				
1. 'Post-mitochondrial'	92923	68	191	0.004
2. 'Microsomal'+cell sap	25893	33	96	0.006
3. 'Microsomal'+pH 5 fraction	32727	38	220	0.011
Expt. 2. With 4 μCi of δ-amino[^{3}H]laevulic acid/ml				
4. 'Post-mitochondrial'	627357	753	1731	0.006
5. 'Microsomal'+cell sap	137107	160	399	0.007
6. 'Microsomal'+pH 5 fraction	49000	1280	2889	0.148
Expt. 3. With 4 μCi of [^{55}Fe]chloride/ml				
7. 'Post-mitochondrial'	55933	590	1309	0.049
8. 'Microsomal'+cell sap	62354	136	386	0.019
9. 'Microsomal'+pH 5 fraction	38716	168	478	0.037
Expt. 4. With 10 μCi of δ-amino[^{3}H]laevulic acid/ml				
10. 'Microsomal'+pH 5 fraction	81686	593	2370	0.062
11. 'Microsomal'+pH 5 fraction	68050	2880	4430	0.130

* Microsomal protein calculated to be present in the incubation.

demonstrating a microsomal site of synthesis for the complete molecule. The values for radioactivity measured are significant by any criterion and certainly higher than those (13.5 and 11 c.p.m.) presented by Kadenbach (1967) as indicating the transfer of cytochrome *c* from the microsomal fraction to mitochondria, and Kadenbach (1970) for the microsomal cytochrome *c* labelled *in vivo*. However, the possibility could remain that a minimum contamination by other proteins would be responsible for such labelling. Several arguments stand against this possibility. The first is that in certain cases not favourable to the synthesis of cytochrome *c* where exactly the same procedure of purification was applied, no counts at all were obtained in cytochrome *c* in spite of the system being perfectly active in the synthesis of other proteins, thus showing the effectiveness of the purification technique.

Secondly, a system that was very active in the incorporation of [^{14}C]leucine or δ-amino[^{3}H]-laevulic acid into cytochrome *c*, the 'microsomal+pH 5.0 fraction', was, however, much less efficient in the synthesis of total protein than the more complex 'post-mitochondrial fraction', which precludes the existence of a contaminant behaving in a reproducible manner along the purification scheme.

Thirdly, it is clear that to assume that the labelling with precursors of the prosthetic group was caused by contaminants, the foreign protein should be a haemoprotein. No such contamination was found spectrophotometrically on our samples. The cytochrome *c* remained radioactive throughout the different variations introduced in the procedure of purification and irrespective of whether a carrier was used for the isolation of the protein. Finally, the low number of counts obtained in cytochrome *c* is not unreasonable since the ratio of its total radioactivity to that accumulated in the rest of the protein is, in two instances, close to the ratio obtained in the intact cell.

We believe therefore that these experiments demonstrate conclusively that the isolated microsomal fraction is able to synthesize the complete molecule of cytochrome *c*, confirming our previous work (González-Cadavid & Campbell, 1967*c*; González-Cadavid *et al.* 1968) and that of Davidian *et al.* (1969), but conflicting with the results of Kadenbach (1970). This disagreement might be due partly to Kadenbach's (1970) failure to extract the newly synthesized cytochrome *c* by the mild

conditions employed, and also to a contaminant of the protein running as apocytochrome *c* in the chromatograms. In this respect it is worthwhile to notice that the total number of counts accumulated in the so-called 'apocytochrome *c*' of the mitochondrial fraction after a 60min incubation of rat liver slices with L-[^{14}C]lysine appears to be nearly four times that obtained in the cytochrome *c* itself. After this prolonged period of incubation, one would expect that the total radioactivity of cytochrome *c* should greatly exceed that of the 'apoprotein' even if this has a high specific radioactivity, since its minimum amount would clearly indicate a rapid transformation into cytochrome *c*.

Another question is whether the cell-free system is in fact able to carry out the net synthesis of cytochrome *c* or whether it is simply effecting chain termination. The low value for incorporation suggests that if initiation really takes place, it must be very much decreased probably owing to the unstability of its mRNA, among other factors. The concentration of Mg^{2+} used in the isolation medium might not necessarily be the optimum for the stability of the polyribosomes engaged in the synthesis of the cytochrome *c* even if it is adequate for the synthesis of other proteins. Further studies should be carried out to elucidate this point and to define the exact requirements of the system to enhance its activity.

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