Synthesis of Haem and Cytochrome *c* Prosthetic Group from δ -Aminolaevulinate by the Cell Sap from Rat Liver

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To determine whether the prosthetic group of cytochrome *c* is synthesized and linked to the apoprotein in the cytosol or in connexion with the endoplasmic reticulum, we have studied the incorporation *in vitro* of δ -amino[^{14}C]laevulinate into porphyrin compounds and cytochrome *c* by the cell sap from rat liver. The radioactive precursor was incorporated into a trichloroacetic acid-precipitable form partially resistant to extractions by acid solvents, suggesting the existence of a fraction covalently linked to protein. The activity was proportional to the amount of protein incubated, did not increase substantially by supplementation with the microsomal fraction and an energy source, and was very low in the pH 5 fraction. Addition of increasing amounts of haemin inhibited the incorporation, as with purified δ -aminolaevulinate dehydratase. [^{14}C]Protoporphyrin IX was identified by paper chromatography, together with a shoulder running as protohaem IX. The cell sap in the absence of ribosomes was also able to incorporate radioactivity into purified cytochrome *c*, and the addition of ribosomes significantly enhanced the activity. The precursors of haem *c* were synthesized in the soluble system by the known haem-synthetic pathway, as shown by the kinetics of labelling of the coproporphyrin, protoporphyrin and haem fractions, and the activities were concentrated in the precipitate obtained between 40 and 60% saturation with $(\text{NH}_4)_2\text{SO}_4$. The presence of ferrochelatase was indicated by the incorporation of ^{55}Fe into proto- and haemato-haem identified by paper chromatography. It is concluded that the cell sap from rat liver contains the complete set of enzymes for the synthesis from δ -aminolaevulinate of haem *c* and its linkage to a small pool of free apoprotein *c* present in soluble form. This suggests that an ancillary pathway of haem synthesis occurs in the cytosol for at least the formation of the prosthetic group, which is linked post-translationally to that pool of apoprotein *c* synthesized by free polyribosomes.

Cytochrome *c* was the first specific mitochondrial protein whose synthesis was shown to occur outside the organelle on cytoplasmic ribosomes, in a study where the kinetics of incorporation of radioactive amino acids into the purified protein was followed in different subcellular fractions from rat liver (González-Cadavid & Campbell, 1967; see González-Cadavid, 1974, for further references). The approach *in vivo* was confirmed by using microsomal, post-mitochondrial, and membrane-free ribosomal systems from regenerating liver, which were able to perform the completion *in vitro* of nascent chains (González-Cadavid *et al.*, 1971; González-Cadavid & Sáez de Córdova, 1974). Most of the experimental evidence is in favour of the assembly of the whole haemoprotein molecule in the cytoplasm, thus indicating that the formation of the thioether bridge between the vinyl chains of haem and the cysteine residues of the apoprotein does not require mitochondrial participation (González-Cadavid, 1974). It is most likely that this is an enzymic process involving the recognition of the steric orientation of both parts of the molecule and their covalent linkage

as an essential step for the acquisition of a stable native conformation (Fisher *et al.*, 1973).

The extramitochondrial location of the holo-protein assembly poses several main questions, such as: (a) whether the activity responsible for the establishment of a thioether linkage is soluble in the cytosol or associated with membranes of the rough endoplasmic reticulum; (b) whether the assembly itself is a ribosomal or post-ribosomal process, the latter implying the possible existence of a soluble pool of free apoprotein; (c) the nature of the immediate precursor of the prosthetic group, among the relative porphyrin derivatives, and the degree of mitochondrial involvement in their synthesis.

Previous work from this laboratory has given some answer to the last question by showing that [^{55}Fe]-haemin can be directly inserted into cytochrome *c* *in vitro* by ribosomal/cell-sap systems (González-Cadavid *et al.*, 1971) and that they can incorporate both ^{55}Fe and δ -amino[^3H]laevulinate into haem *c* in the complete absence of mitochondria (González-Cadavid *et al.*, 1971; González-Cadavid & Sáez de

Córdova, 1974). However, the existence of a complete cytoplasmic pathway for haem synthesis from δ -aminolaevulinate does not seem to fit with the accepted scheme of the subcellular compartmentation of enzymes involved in haem synthesis. Thus coproporphyrinogen oxidase and ferrochelatase, catalysing the two last steps, namely the conversion of coproporphyrinogen III into protoporphyrin IX and finally into protohaem IX, have not been detected in significant amounts in liver cytoplasm (Sano & Granick, 1961).

To examine these questions we have studied the relative contribution of the endoplasmic reticulum and the cytosol to the incorporation *in vitro* of δ -amino[^{14}C]laevulinate into porphyrin compounds and cytochrome *c* and followed the sequential transfer of radioactivity from coproporphyrins to protoporphyrins and haem as well as ^{55}Fe insertion into the latter. Our results show that the cell sap from rat liver contains a soluble pool of free apoprotein *c*, a system responsible for its linkage to the prosthetic group, and the enzymes necessary for haem synthesis from δ -aminolaevulinate.

Materials and Methods

Chemical and animals

$^{55}\text{FeCl}_3$ (sp. radioactivity 8 Ci/g of Fe), L-[U- ^{14}C]leucine (sp. radioactivity 297 mCi/mmol) and δ -amino[^{14}C]laevulinate hydrochloride (sp. radioactivity 59 mCi/mmol) were purchased from the Commissariat à l'Énergie Atomique (CEA), Gif-sur-Yvette, France. Protoporphyrin IX (grade B) and haematoporphyrin IX dihydrochloride were from Calbiochem, San Diego, CA, U.S.A., and haemin type I (bovine) was from Sigma Chemical Co., St. Louis, MO, U.S.A. All other materials were as specified by González-Cadavid & Sáez de Córdova (1974) and González-Cadavid *et al.* (1971).

Partially hepatectomized male rats (48 h liver regeneration) were used throughout, except in a few experiments where normal rats (150 g body wt.) were used. All animals were starved for 18 h before death. For other details see González-Cadavid *et al.* (1971).

Preparation and incubation of subcellular fractions

All fractionations were carried out at 2–4°C. The microsomal, Millipore-filtered cell sap and pH 5 fractions were prepared in medium B, containing 10 mM- MgCl_2 , 35 mM-Tris/HCl buffer, pH 7.8, at 20°C, 25 mM-KCl and 0.15 M-sucrose, as indicated by González-Cadavid & Sáez de Córdova (1974). $(\text{NH}_4)_2\text{SO}_4$ fractionation of the cell sap was carried out by sequential addition of the solid salt with continuous stirring at 0°C and pH 7.0, up to 20, 40, 60, 80 and 100% saturation. The precipitates were collected by

centrifugation at 18000g for 15 min. The pellets were dissolved in 2 ml of medium B and, as with the final supernatant from 100% saturation, were dialysed against the same medium to remove $(\text{NH}_4)_2\text{SO}_4$. Protein contents were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Incubations were performed at 37°C in a metabolic incubator at approx. 100 oscillations/min in either tubes (0.5 ml final volume) or 50 ml Erlenmeyer flasks (5 ml final volume), under dim light and free aeration. The composition of the standard incubations under conditions for protein synthesis were as follows: microsomal RNA (0.25 mg/ml); cell sap or pH 5 fraction (as indicated in each experiment); sucrose (90 mM); Mg^{2+} (6 mM); K^+ (25 mM); Tris/HCl buffer, pH 7.8 at 20°C (21 mM, or 39 mM when the pH 5 fraction was used); ATP (2 mM); phosphoenolpyruvate (10 mM); pyruvate kinase (50 $\mu\text{g}/\text{ml}$); GTP (0.25 mM); and radioactive precursor as indicated. When the microsomal particles were omitted (incubations of soluble fractions alone), no energy source was present either. Variations to this basic system are indicated in the Figures. The incorporation of radioactivity was stopped by immersing the tubes in an ice bath and adding the corresponding non-radioactive precursor to a final concentration of 10 mM.

Measurement of radioactivity in cytochrome *c*

Labelled cytochrome *c* was isolated from the 5 ml incubations by applying a procedure involving salting out with $(\text{NH}_4)_2\text{SO}_4$, ion-exchange chromatography on Amberlite CG-50 and polyacrylamide-gel electrophoresis, as previously described (González-Cadavid & Sáez de Córdova, 1974). Radioactivity in the holoprotein was determined as indicated in that paper. The electrophoresis buffer was 0.1 M- K_2HPO_4 (pH 7.2)/0.1% sodium dodecyl sulphate. After the runs, the gels were scanned at 280 nm in a Joyce-Loebl u.v. densitometer type D8MK2, and the yield of carrier cytochrome *c* was estimated gravimetrically by weighing the paper area under the peaks. This value was used for normalizing the radioactivity counted on the gel to the carrier cytochrome *c* added initially to the samples. The radioactivity of the main and the satellite peaks of cytochrome *c* in the polyacrylamide gels was either computed from the sum of the respective fractions counted individually, or determined directly on the pool of five to seven slices corresponding to each peak as described by González-Cadavid & Sáez de Córdova (1974).

Measurement of radioactivity in mixtures of porphyrin compounds

An indirect estimation of the δ -amino[^{14}C]laevulinate incorporated into porphyrin compounds

free of contamination by the 10% trichloroacetic acid-soluble precursor was obtained by applying the glass-fibre-disc method of Mans & Novelli (1961) as described by González-Cadavid & Sáez de Cordova (1974). The only modification was that heating in 5% trichloroacetic acid was omitted. In separate tests [^{14}C]haemin or δ -amino[^{14}C]laevulinate adsorbed to the filters was washed out completely under these conditions. The radioactivity determined by this procedure represents the porphyrin compounds linked to protein in a linkage resistant to conc. HCl/acetone (1:19, v/v), and is approx. 20–25% of the total incorporation in tetrapyrroles (see the Results section).

A more representative determination of [^{14}C]porphyrin compounds was based on precipitation of the incubations with 10% trichloroacetic acid and centrifugation at 30000g for 10 min. After three washings by resuspension in 5% trichloroacetic acid, the sediment was finally extracted three times with conc. HCl/acetone (1:19, v/v). The supernatants were pooled, diluted with 6 vol. of water and extracted with 2 vol. of diethyl ether. The ether phase was washed once with 5% NaCl, twice with water and finally evaporated to dryness under a N_2 current. The residue was dissolved in 0.1 M-Tris base and adjusted to pH 8.0 with 2 M-HCl. Portions (10 μl) were used for counting of radioactivity with the T-21 fluid (Patterson & Greene, 1965) and determination of the pyridine haemochromogen spectrum (Fuhrhop & Smith, 1975).

Fractionation of radioactive porphyrins and ferro-porphyrins

The procedure of Rimington *et al.* (1963) as used by Van der Merwe & Findlay (1965) was applied with several modifications, as follows. A 2 ml sample from the incubation was diluted with 2 ml of water and mixed with 15 ml of ethyl acetate/acetic acid (4:1, v/v), and shaken for 5 min at 2–4°C. The two phases were separated by centrifugation at 10000g for 5 min and the upper phase was collected. The water phase was re-extracted with 5 ml of the same solvent and the upper phases were pooled. This extract was washed twice with an equal volume of 3% (w/v) sodium acetate and the washings were extracted with a small volume of ethyl acetate. The upper phases were pooled and then shaken with 2 ml of 10% (w/v) HCl in four successive steps to remove porphyrins. The ethyl acetate phase contains haems, and is termed fraction F-3. The HCl extract was adjusted to pH 3 with solid sodium acetate and 1 ml of acetic acid was added to lessen emulsifying. The porphyrins were extracted by shaking twice with 10 ml of diethyl ether, and the ether phases were combined and washed once with 2 ml of 1% Na_2CO_3 and twice with 2 ml of water.

The ether extract was treated with 1 ml of 0.12 M-HCl which removes the coproporphyrins, and the product was named fraction F-1. Finally, the protoporphyrins were extracted from the ether by shaking twice with 0.5 ml of 25% (w/v) HCl. This fraction was named F-2. For determinations of radioactivity a 0.1 ml sample of fraction F-2 was diluted to 1 ml with water and mixed with 10 ml of Instagel. The total F-1 fraction (approx. 1 ml) was counted for radioactivity with 10 ml of Instagel. For fraction F-3, a 1 ml portion was mixed with 10 ml of T-21 fluid. Zero-time values of radioactivity were subtracted in each case.

Chromatography of fractions F-2 and F-3 was performed by the technique of Nicholas & Rimington (1951) as described by Fuhrhop & Smith (1975), by using a descending system of water-saturated 1,4-lutidine. After 18 h runs the spots were located by u.v. light, the paper was sectioned into strips corresponding to each sample and then they were cut into 25–30 equal rectangles that were counted directly as the glass-fibre discs.

Results

Synthesis of protein-linked porphyrin compounds from δ -aminolaevulinate

The microsomal fraction from regenerating liver depends, as with all ribosomal systems, on the presence of soluble factors for functioning in protein synthesis. The plateau of [^{14}C]leucine incorporation into protein in 0.5 ml incubation mixtures (Fig. 1a) was reached at about 75 μl of pH 5 fraction (0.5 mg of protein) for 0.125 mg of microsomal RNA, whereas for the cell sap the maximum value was obtained with 25 μl (0.6 mg of protein), decreasing thereafter. At the optimum amounts of both fractions, the radioactivity incorporated in the presence of cell sap was only two-thirds of that obtained with the pH 5 fraction. This is well known, and it is probably due to the presence of free amino acid pools in the cell sap which dilute the added radioactivity, plus some other factors absent from the pH 5 fraction.

Both soluble fractions were also required for the incorporation of the porphyrin precursor δ -aminolaevulinate into a trichloroacetic acid-precipitable acid/acetone-resistant material (Fig. 1b). In striking contrast with protein synthesis, the cell sap was far more active than the pH 5 fraction. The radioactivity curve did not reach a maximum but rather was shaped like the lower part of a sigmoid plot, suggesting a co-operative process involving an increase in the active enzyme system and of the apoprotein pools that bind the recently synthesized porphyrins. At 100 μl , the ratio between incorporation by the cell sap and the pH 5 fraction was higher than 6.

On scaling up the incubation systems to 5 ml, appropriate for the experiments on cytochrome *c*,

Fig. 1. Incorporation of [^{14}C]leucine and δ -amino[^{14}C]laevulinate into a trichloroacetic acid-precipitable acid/acetone-resistant linkage, by the microsomal fraction in the presence of increasing concentrations of soluble fractions

The microsomal fraction from regenerating liver was incubated for 30 min in an energy-supplemented system with increasing amounts of cell sap (○) or pH 5 fractions (●) from the same source, either with $0.2 \mu\text{Ci}$ of [^{14}C]leucine/ml (a) or $0.2 \mu\text{Ci}$ of δ -amino[^{14}C]laevulinate/ml (b), in a total volume of 0.5 ml. The radioactivity was determined by the glass-fibre-disc method, only in the material retained on the discs. Results are means of three separate experiments performed in duplicate $\pm$ range of ratios calculated within each experiment. Mean values of maximum incorporation were 24650 d.p.m. ([^{14}C]leucine, pH 5 fraction) and 9900 d.p.m. (δ -amino[^{14}C]laevulinate, cell sap) for the 0.5 ml incubation. Original protein concentrations were 23 mg/ml for cell sap and 6.7 mg/ml for pH 5 fraction.

1 ml of soluble fractions was used. The incubations were carried out for 30 min with an energy source and $1 \mu\text{Ci}$ of δ -amino[^{14}C]laevulinate/ml, either in the presence or absence of microsomal fraction. Mean values ($\pm$ s.d.) of four separate experiments performed in duplicate were 298000 ± 36000 d.p.m./5 ml for the microsomal-cell sap system. The ribosomes are not essential, since the cell sap by itself incorporated radioactivity to $80 \pm 10\%$ of the complete system. This proved that the activity was unrelated to the functioning of protein synthesis, in agreement with the demonstration of the much lower incorporation obtained with the pH 5 fraction in the presence or the absence of the microsomal fraction ($29 \pm 6\%$ and $8 \pm 4\%$ respectively of the radioactivity incorporated by the cell sap-microsomal system). A further support for this assumption relies on the fact that removal of the energy source not only did not inhibit but even increased the incorporation in all systems (results not shown).

The procedure used so far for measuring δ -aminolaevulinate incorporation was simply the glass-fibre-disc method usually applied for preparing protein for radioactivity counting, except that no heating in 5% trichloroacetic acid was introduced and that the material was submitted to two washings with acid/acetone before the ether treatment. This would leave out the free or loosely bound tetrapyrrolic compounds, but at the same time remove the δ -amino[^{14}C]laevulinate which otherwise would require a much more laborious solvent-extraction procedure.

To confirm that the radioactivity was not due to adsorption or unspecific covalent binding of δ -amino[^{14}C]laevulinate to the material on the filters, the incubations of cell sap were carried out in the presence of increasing concentrations of exogenous haemin, which should not decrease the radioactivity if the latter is merely due to δ -aminolaevulinate. The addition of haemin inhibited the incorporation by 95% (Fig. 2), thus ruling out contamination with δ -aminolaevulinate and at the same time indicating that some specific enzymic step was being affected. The sigmoid curve obtained was strikingly similar to that of the feedback inhibition of δ -aminolaevulinate dehydratase induced by the addition of haemin to the purified enzyme (Callisano *et al.*, 1966), indicating that the radioactivity was present in a compound formed after this step in the haem-synthetic pathway, that is from porphobilinogen onwards. The inhibition was not unexpected, because δ -aminolaevulinate dehydratase is a cytoplasmic enzyme, but we had to be sure that the compound formed was indeed a tetrapyrrole to accept the possibility of a precursor relationship to the cytochrome *c* prosthetic group. Whatever the compound was, iron would be finally present, thus implying the presence of ferrochelatase activity. The precise identification of the final product of synthesis would be difficult in the material resistant to acid/acetone extraction, and we had therefore to analyse only those porphyrins in loose binding split by the acid-solvent treatment.

The acid/acetone and acid/ethanol/ether washings of the glass-fibre discs were shown to remove in

Fig. 2. Inhibition by exogenous haemin of δ -amino[^{14}C]-laevulinate incorporation by the cell sap into a trichloroacetic acid-precipitable acid/acetone-resistant linkage. The cell sap from normal liver (0.1 ml) was incubated for 30 min in 0.5 ml final volume without an energy source in the presence of 0.2 μCi of δ -amino[^{14}C]-laevulinate/ml and increasing concentrations of added haemin. The radioactivity was determined both in the acid-ethanol/ether washings and in the material remaining on the filters. The percentages plotted correspond to the total radioactivity calculated by addition of both separate values relative to the controls without exogenous haemin (mean value 29 600 d.p.m.).

Fig. 3. Chromatographic identification of radioactive porphyrin compounds in a crude extract from cell sap incubations

The cell sap from normal liver (4 ml) was incubated for 30 min with 20 μCi of δ -amino[^{14}C]laevulinate in a total volume of 20 ml, without an energy source. The suspension was precipitated with 5% trichloroacetic acid, and submitted to the procedure described for obtaining a 'crude' mixture of porphyrin derivatives in 50 μl of 0.1 M-Tris/HCl, pH 8.0. A sample of 20 μl was analysed by paper chromatography with a mixture of 2,4- and 2,5-lutidine saturated with water. — $\rightarrow$ R_F values of the three radioactivity peaks; ---- $\rightarrow$ positions of external markers in the following order: δ -amino[^{14}C]laevulinate; haemin Cl; protoporphyrin IX; and haematoporphyrin IX. The spots identified in the sample were only two, one corresponding to haemin Cl, and another with an R_F of 0.96.

total about 65% of the trichloroacetic acid-insoluble radioactivity, irrespective of the washing sequence. The discs themselves are not responsible for the residual radioactivity, since acid/acetone treatment of pellets obtained by trichloroacetic acid precipitations left about 30% insoluble radioactivity. Nor does trichloroacetic acid seem to be the cause of the resistance to acid/acetone since direct washing of the discs by this solvent, omitting the trichloroacetic acid treatment, left unextracted nearly 25% of the calculated radioactivity.

The identification of the synthesized compounds was therefore carried out on the acid/acetone extract of the trichloroacetic acid precipitate, which was then submitted to partition in water/ether systems. The pyridine haemochromogen spectrum showed a perfect coincidence with that of haemato-haemin used as standard and with the literature data (Fuhrhop & Smith, 1975). But this, of course, did not prove that the radioactivity was indeed associated with haem, and a chromatographic separation of the labelled compounds was performed in a descending lutidine/water system. Fig. 3 shows that the radioactivity is present in tetrapyrroles with two carboxylic side chains, as can be inferred from their R_F values between 0.7 and 0.8 (see Nicholas & Rimington, 1951). The main peak has an R_F coinciding with that of protoporphyrin IX run as external standard, but

the presence of overlapping radioactive haem cannot be excluded, since ferroporphyrins run in this system with an R_F somewhat lower than that of their respective porphyrins. Alternatively, it is also possible that the shoulder of radioactivity having an R_F of 0.70, and running as the standard haemin, corresponds to protohaem IX. The minor ^{14}C zone present in the middle of the chromatogram (R_F 0.46) is probably due to residual δ -aminolaevulinate.

*Incorporation of δ -aminolaevulinate acid into the prosthetic group of cytochrome *c**

The cell sap from regenerating liver is able not only to synthesize porphyrins from δ -amino[^{14}C]laevulinate but even to incorporate the radioactivity into cytochrome *c*. This was shown by a series of experiments where cytochrome *c* was isolated and purified by a combination of ion-exchange chromatography, salting out and polyacrylamide-gel electrophoresis. The radioactivity in the microsomal+cell sap system was almost exclusively present in the 15% acrylamide

Fig. 4. Incorporation of δ -amino[^{14}C]laevulinate into the prosthetic group of cytochrome *c* by subcellular soluble fractions under conditions of protein synthesis

Cytochrome *c* was extracted from the 5 ml incubations described in the Results section, purified by ion-exchange chromatography and salting out and finally submitted to electrophoresis in the presence of 0.1% sodium dodecyl sulphate in 'tandem' gels made with 10 and 15% acrylamide. After densitometry at 280 nm the gels were cut into 2 mm fractions and the radioactivity was determined. Only the 15%-acrylamide sections of the gels are depicted. (a) Cell sap with microsomal fraction; (b) cell sap without microsomal fraction; (c) pH5 fraction with microsomal fraction; (d) pH5 fraction without microsomal fraction. —, A_{280} of carrier cytochrome *c*; ○—○, radioactivity.

section of the gels (Fig. 4a), and there it was associated with the main A_{280} peak, corresponding to carrier cytochrome *c* added for the isolation procedure. The two higher-molecular-weight satellite peaks (visible also with the naked eye), which were also radioactively labelled, are probably polymeric forms arising as artifacts of purification.

The cell sap alone, free of ribosomes or subunits, incorporated a substantial amount of ^{14}C into the cytochrome *c* peak (Fig. 4b), although the calculated specific radioactivity was about 35% lower than in the presence of the microsomal fraction. This strongly suggests that a free cytochrome *c* apoprotein pool is present in the cell sap that binds to the ^{14}C -labelled prosthetic group and explains why cytochrome *c* becomes radioactive in the absence of protein synthesis. The pH5 fraction was much less active than the cell sap, in keeping with the low haem-synthesizing activity of the former and probably due also to the loss of the free apoprotein in the pH5 supernatant (Figs. 4c and 4d).

Kinetics of haem synthesis by the cell sap

A preliminary fractionation of the porphyrin-synthesizing system of the cell sap was carried out by $(\text{NH}_4)_2\text{SO}_4$ precipitation, as shown in Fig. 5. The precipitate formed between 40 and 60% saturation with the salt (P-60) had 25% of the protein and a 1.7-fold higher specific radioactivity than the original cell sap. This P-60 fraction incorporated δ -amino[^{14}C]laevulinate into the trichloroacetic acid-precipitable acid/acetone-resistant form in a linear fashion up to 2 h (22000 d.p.m./mg of cell-sap protein), and reached a plateau between 3 and 4 h of incubation.

The chromatographic separation of a crude mixture of ^{14}C -labelled porphyrin compounds applied in the experiment of Fig. 3 suggested the presence of radioactive protohaem IX, but did not allow a conclusive identification. For this purpose we decided to combine three approaches as follows: (a) simultaneous incubation with ^{55}Fe and δ -amino-

Fig. 5. Preliminary fractionation of the porphyrin-synthesizing activity present in the cell sap

A 5 ml sample of cell sap from regenerating liver (17.1 mg of protein/ml) was fractionated by $(\text{NH}_4)_2\text{SO}_4$ as described in the text. Incubations were carried out for 30 min at 37°C in a medium containing soluble protein (4 mg/ml; fraction S-100, 0.4 mg/ml); Mg^{2+} (10 mM), K^+ (25 mM), Tris (35 mM)/HCl, sucrose (150 mM) and δ -amino ^{14}C laevulinate (0.25 $\mu\text{Ci/ml}$), all at pH 7.8 (20°C) and in a total volume of 0.5 ml. Radioactivity was estimated by the glass-fibre-disc method on the material remaining on the discs. The value for the unfractionated cell sap was 2713 d.p.m./mg of protein. The plot was constructed as described by de Duve (1967), where the fractional amount of protein is the percentage amount of protein present in the fraction, and the relative specific radioactivity is the specific radioactivity in the fraction divided by the specific radioactivity of the sum of fractions and measures the degree of purification achieved. The area of each rectangle represents the percentage of total activity present in each fraction. Fractions P20–P100 are precipitates obtained by adding $(\text{NH}_4)_2\text{SO}_4$ at the saturation percentages indicated by the numbers; S100 is the final supernatant from 100% saturation.

Fig. 6. Kinetics of porphyrin synthesis by the P-60 fraction from cell sap

The P-60 fraction was incubated as in the experiment of Fig. 5 in a total volume of 5 ml, in duplicate. Samples (0.1 ml) were withdrawn at the times indicated and used for the measurement of radioactivity by the glass-fibre-disc method. A 2.5 ml sample was used for the fractionation of porphyrin compounds as described in the text. The numbers on top of the blocks for each period are radioactivity values in d.p.m. $\times 10^{-3}$ calculated for the total volume of incubation. ■, Fraction F1 (coproporphyrins); □, fraction F2 (porphyrins); ▨, fraction F3 (haemins).

δ -amino ^{14}C laevulinate and selecting two periods with a low and higher labelling of the haem fraction. At 30 min (Fig. 7a), the radioactivity present in the haem fraction was found in two regions of the chromatogram, with R_F values of 0.74 and 0.85, corresponding to protohaem IX and haematoxaem respectively. (A smaller peak with an R_F of 0.45 was probably δ -aminolaevulinate.)

At 2 h incubation (Fig. 7b), all the ^{14}C and ^{55}Fe were present in the haematoxaem peak, thus confirming the iron porphyrin synthesis inferred from the results obtained in the crude tetrapyrrole mixture analysed in Fig. 3.

The ability of the liver cell sap to carry out haem synthesis from δ -aminolaevulinate does not seem to derive from leakage of coproporphyrinogen oxidase and ferrochelatase from damaged mitochondria. This was suggested by an experiment where essentially similar radioactivities were obtained in the trichloroacetic acid-insoluble acid/acetone-resistant material prepared from incubations of cell sap, isolated by increasing degrees of tissue homogenization leading to concomitant extents of mitochondrial damage. On the other hand, the fact that Sano & Granick (1961) were unable to detect soluble coproporphyrinogen oxidase is probably due to some particular feature of the incubation method used by these authors for measuring protoporphyrinogen formation from coproporphyrinogen. Preliminary experiments show that the cell sap obtained and incubated under the conditions used by Sano & Granick has

^{14}C laevulinate to detect ferrochelatase activity; (b) fractionation of the crude mixture of porphyrins before chromatography in order to avoid overlapping of highly labelled compounds; (c) study of the labelling kinetics of haem precursors to determine whether the known precursor/product relationships were found. In a preliminary experiment, the P-60 fraction was incubated for several periods with δ -amino ^{14}C laevulinate only and the radioactivity was determined in each of the extracted fractions. Fig. 6 shows that at 30 min of incubation the ^{14}C content was approximately similar in the coproporphyrin and protoporphyrin fractions, with only negligible labelling of the haemins. In later periods, radioactivity considerably decreased in coproporphyrin, rising in protoporphyrin (peak at 1.5–2 h), and finally in haemin (peak at 4 h).

For chromatographic identification, the experiment was repeated with cell sap, by using ^{55}Fe and

Fig. 7. Incorporation *in vitro* of ^{55}Fe into protohaem IX and haematoaem by the cell sap

The cell sap from regenerating liver was incubated in a total volume of 2 ml for either 30 (a) or 120 (b) min with $0.25\ \mu\text{Ci}$ of δ -amino ^{14}C laevulinate/ml and $2\ \mu\text{Ci}$ of ^{55}Fe /ml, as in Fig. 5. Protein concentration was $4\ \text{mg/ml}$. Porphyrin compounds were fractionated as described in the text and fraction F3 (haems) was dissolved in 30 ml of ethyl acetate/acetic acid (4:1, v/v). Samples of $25\ \mu\text{l}$ (a) and $10\ \mu\text{l}$ (b) were subjected to paper chromatography. Arrows indicate the R_F for the peaks of radioactivity. ^{14}C ; $\circ$, ^{55}Fe ; $\cdots$, calculated peaks in overlapping section.

10–15-fold lower activity than the system described in the present paper, as judged from the radioactivity in all the fractions extracted.

Discussion

In this work we tried to solve three main points. The first question was whether cytoplasmic systems free of mitochondrial contamination were indeed able to synthesize the cytochrome *c* prosthetic group from δ -aminolaevulinate *in vitro* and to link it to its apoprotein. Our results not only confirmed our previous data with the post-mitochondrial fraction (González-Cadauid *et al.*, 1971; González-Cadauid & Sáez de Córdoba, 1974) but also demonstrated that the activities reside mainly in the soluble part of the system, that is in the cell sap, and that the membrane or ribosomal constituents contribute only slightly to haem synthesis and its linkage to total protein. This is reasonable, because the soluble proteins albumin and haemopexin which bind haem both *in vivo* and *in vitro* (Negishi & Omura, 1970; Marecek *et al.*, 1973; Correia & Meyer, 1975) are present in fairly

large amounts in the cell sap, so that the number of chains completed *in vitro* by the ribosomes is negligible in comparison with the total pool of binding proteins.

In the specific case of cytochrome *c*, the fact that the cell sap can incorporate a substantial amount of δ -amino ^{14}C laevulinate into the prosthetic group in the absence of protein synthesis (no energy supplementation or ribosomes) points to the existence of a soluble pool of the free apoprotein, which hitherto had not been demonstrated. It is likely that when the cell sap is complemented with ribosomes under conditions of protein synthesis there is also some contribution of growing peptide chains of the cytochrome *c* apoprotein available for linkage to the prosthetic group. The dilution factor of the recently synthesized apoprotein by the pre-existing soluble pool cannot be determined without an accurate estimate of the specific radioactivity of free haem under the two sets of conditions, that is in the presence or absence of ribosomes. The existence of a small but significant pool of apoprotein *c* in the cytosol would agree with findings of other authors about microsomal cytochromes, such as *b₅* (Negishi & Omura, 1970) or *P-450* (Correia & Meyer, 1975).

The second point of interest was to demonstrate the existence in the cell sap of an active system, most likely enzymic in nature, responsible for the formation of the thioether bridge between the two sections of the cytochrome *c* molecule. This opens a way for studying *in vitro* the mechanism of linkage by suitable separation and recombination of the different components involved, namely a haem-generating complex, a free apoprotein *c* pool, and the ligand activity. An initial approach was the isolation of a cell-sap fraction, named P-60, which was able to perform all the steps of haem synthesis from δ -aminolaevulinate and which supplemented with apoprotein *c* should be applicable for the detection of the ligase activity among the cell-sap proteins.

In turn, reconstituted binary systems containing the apoprotein *c* and the ligase should serve for the identification of the immediate precursor of haem *c*, that is whether haem is inserted directly or as a porphyrin where iron is added in a subsequent stage. Our present results show that coproporphyrins, protoporphyrin IX, protohaem and haematoaem are formed, but the only hints as to which of these compounds is linked to apoprotein *c* come from previous work where ^{59}Fe cytochrome *c* was isolated from incubations of liver microsomal fractions supplemented with cell sap (González-Cadauid *et al.*, 1971), and from results with the bacterium *Spirillum itersoni* (Lascelles *et al.*, 1969; Jones & Jones, 1970) incubated with ^{59}Fe haem. Similarly, there is evidence that protoporphyrinogen IX is not the immediate precursor of haem *c* (Garrard, 1972), in contrast with the assumptions of Sano & Tanaka (1964).

Finally, the third question concerns the relevance of the present work to the situation *in vivo*. Our results with tissue disruption performed at different degrees of homogenization make it unlikely that the coproporphyrinogen oxidase and ferrochelatase activities present in the cell sap arise from mitochondrial leakage. This agrees with the tight association of these enzymes with mitochondrial membranes, requiring rather drastic conditions for their isolation (Sano & Granick, 1961; Jones & Jones, 1970). On the other hand, there are reports on the existence of soluble ferrochelatase in *Rhodopseudomonas spheroides* (Jones & Jones, 1970), and of a soluble extra-mitochondrial component of the coproporphyrinogen oxidase system in *Saccharomyces cerevisiae* (Poulson & Polglase, 1974).

The other mitochondrial enzyme, δ -aminolaevulinic synthetase, has also been found in the cytoplasm (Hayashi *et al.*, 1972; Scholnick *et al.*, 1972) and even isolated and purified in an active form from the cell sap (Scholnick *et al.*, 1972). Some results obtained *in vivo* showing a higher specific radioactivity of cytoplasmic haem in rat liver (Yoda & Israels, 1972) after the intraperitoneal injection of δ -amino[^{14}C]-laevulinate may well indicate synthesis *in situ* besides the one-way transfer of early-labelled haem from the mitochondria. Taking all this evidence together, it would not be too speculative to assume that in the liver cell, as well as the accepted pathway for haem synthesis, where the first and last two steps are mitochondrial, there is an alternative pathway where all the enzymes are located in the cytoplasm. The main objection is of course the apparent absence of succinyl-CoA (3-carboxypropionyl-CoA) or succinyl-CoA-generating system in the cytoplasm of mammalian cells.

If haem and porphyrin can be synthesized by two alternative but perhaps co-operative systems, the question arises as to the relative importance of each process and the physiological meaning of haem compartmentation. At least for the cytochrome *c* prosthetic group it is tempting to postulate a significant participation of the cytosolic pathway in the synthesis and linkage of haem to the free cytochrome *c* apoprotein pool. If one assumes that the cytochrome *c* peptide chain made on membrane-bound polyribosomes (González-Cadavid & Sáez de Córdova, 1974) is vectorially discharged in the lumen of microsomal vesicles, the soluble apoprotein *c* detected in the cytosol would correspond only to that fraction of cytochrome *c* formed on free polyribosomes. In this case, the prosthetic-group linkage should necessarily occur at a post-translational level.

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References

- Callisano, P., Bonsignore, D. & Cartasegna, C. (1966) *Biochem. J.* **101**, 55–552
- Correia, M. A. & Meyer, V. A. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 400–404
- de Duve, C. (1967) in *Enzyme Cytology* (Roodyn, D. B., ed.), pp. 1–26, Academic Press, London and New York
- Fisher, W. R., Taniuchi, H. & Anfinsen, C. B. (1973) *J. Biol. Chem.* **248**, 3188–3195
- Fuhrhop, J. H. & Smith, K. M. (1975) *Laboratory Methods in Porphyrin and Metalloporphyrin Research*, pp. 83–87, Elsevier Scientific Publishing Co., Amsterdam
- Garrard, W. T. (1972) *J. Biol. Chem.* **247**, 5935–5943
- González-Cadavid, N. F. (1974) *Sub-Cell. Biochem.* **3**, 275–309
- González-Cadavid, N. F. & Campbell, P. N. (1967) *Biochem. J.* **105**, 443–450
- González-Cadavid, N. F. & Sáez de Córdova, C. (1974) *Biochem. J.* **140**, 157–167
- González-Cadavid, N. F., Ortega, J. & González, M. (1971) *Biochem. J.* **124**, 685–694
- Hayashi, N., Kurashima, Y. & Kikuchi, G. (1972) *Arch. Biochem. Biophys.* **148**, 10–21
- Jones, M. S. & Jones, O. T. G. (1970) *Biochem. J.* **119**, 453–462
- Lascelles, J., Rittenberg, B. & Clark-Walker, G. D. (1969) *J. Bacteriol.* **97**, 455–456
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Mans, R. J. & Novelli, G. D. (1961) *Arch. Biochem. Biophys.* **94**, 48–53
- Marecek, Z., Jirsa, M. & Korinek, J. (1973) *Clin. Chim. Acta* **45**, 409–413
- Negishi, G. & Omura, T. (1970) *J. Biochem. (Tokyo)* **67**, 745–747
- Nicholas, R. E. H. & Rimington, C. (1951) *Biochem. J.* **48**, 306–313
- Patterson, M. S. & Greene, R. C. (1965) *Anal. Chem.* **37**, 854–857
- Poulson, R. & Polglase, W. J. (1974) *FEBS Lett.* **40**, 258–260
- Rimington, C., Morgan, P. N., Nicholls, K., Everall, J. D. & Davies, R. R. (1963) *Lancet* **ii**, 318–322
- Sano, S. & Granick, S. (1961) *J. Biol. Chem.* **236**, 1173–1180
- Sano, S. & Tanaka, K. (1964) *J. Biol. Chem.* **239**, PC3109–PC3110
- Scholnick, P. L., Hammaker, L. E. & Marver, H. S. (1972) *J. Biol. Chem.* **247**, 4126–4131
- Van der Merwe, L. & Findlay, G. H. (1965) *S. Afr. J. Med. Sci.* **30**, 49–56
- Yoda, B. & Israels, L. G. (1972) *Can. J. Biochem.* **50**, 633–637