The Biosynthesis of Cytochrome *c*

SEQUENCE OF INCORPORATION *IN VIVO* OF [¹⁴C]LYSINE INTO CYTOCHROME *c* AND TOTAL PROTEINS OF RAT-LIVER SUBCELLULAR FRACTIONS

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1. In order to determine the initial intracellular site of synthesis of cytochrome *c* in the liver cell, groups of rats were injected with [¹⁴C]lysine and killed 7.5, 15, 30 and 60 min. later. The livers were homogenized in 0.3M-sucrose and subcellular fractions obtained. The mitochondrial fraction was further subfractionated. Pure cytochrome *c* was isolated from extracts of each fraction, obtained first with water at pH 4.0 and then with 0.15M-sodium chloride. 2. A comparison of the kinetics of incorporation of [¹⁴C]lysine into total protein for each particulate fraction showed the usual two different kinds of kinetics. Incorporation into all the mitochondrial subfractions and the nuclear fraction rose gradually to a plateau value at about 20 min., in contrast with that into the two microsomal fractions which rose rapidly to a peak value about seven times that for the mitochondrial fractions. The kinetics for the incorporation into mitochondrial cytochrome *c* showed a plateau value at 30 min. about three times that for the total mitochondrial protein. There was no difference in the specific radioactivity of the mitochondrial cytochrome *c* extracted with water or 0.15M-sodium chloride or between the different mitochondrial subfractions. In contrast, the cytochrome *c* isolated from water extracts of the microsomal fractions had a lower specific radioactivity than that obtained from the 0.15M-sodium chloride extract. The specific radioactivity of the latter showed a rapid rise to a peak value about four times that for the mitochondrial cytochrome *c*, and the shape of the curve was similar to that for the total protein of the microsomal fraction. The results suggest that cytochrome *c* is synthesized *in toto* by the morphological components of the microsomal fraction. It seems first to be bound tightly to a microsomal particle, passing then to a looser microsomal binding and being finally transferred to the mitochondria. The newly synthesized cytochrome *c* in the mitochondrion could not be differentiated from the old by its degree of extractability at pH 4.0.

The fact that Roodyn, Suttie & Work (1962) were unable to demonstrate the synthesis of cytochrome *c* and malate dehydrogenase by isolated preparations of rat liver mitochondria raised the question of the site of biosynthesis of the soluble proteins of the mitochondria in the cell. Though it remained possible that the failure of a system *in vitro* was without significance for the physiology of the intact cell, the idea arose that some mitochondrial protein might be synthesized by an extramitochondrial component of the cytoplasm. Since previous work in this Laboratory has been concerned with the role of the microsomal fraction from rat liver in the synthesis of specific proteins, a study of the possible synthesis of cytochrome *c* by a component of this fraction was undertaken.

The choice of cytochrome *c* as a model for the synthesis of mitochondrial soluble proteins was dictated by various considerations. Most of the cytochrome *c* of the cell is located in the mitochondria, and is relatively easily extracted, estimated and purified. Moreover, as an example of a haemoprotein whose primary structure is established, coupled with its key role in the electron-transport chain, the biosynthesis of cytochrome *c* is an interesting study in itself. Marsh & Drabkin (1957) have demonstrated previously that there is an active synthesis of cytochrome *c* by the liver of the adult rat.

In the present paper the kinetics of the incorporation *in vivo* of [¹⁴C]lysine into cytochrome *c* and total protein have been studied to establish the

initial site of cytochrome *c* synthesis among the usual subcellular fractions. This work is based on a study of the distribution of cytochrome *c* among the subcellular fractions and the methods developed for the isolation of pure cytochrome *c* from these fractions (González-Cadauid & Campbell, 1967c). A preliminary report of some aspects of the present investigation has appeared previously (González-Cadauid & Campbell, 1967a).

While this work was in progress, Beattie, Basford & Koritz (1966) reported that the kinetics of the incorporation of [^{14}C]valine in rat kidney *in vivo* indicated a much higher rate of synthesis of soluble, as opposed to structural, mitochondrial proteins. The results were similar but less striking for rat liver. The evidence is consistent with the extramitochondrial synthesis of cytochrome *c*. In a preliminary report of studies on the site of synthesis of cytochrome *c* by Krebs mouse ascites-tumour cells, Haldar, Freeman & Work (1966) have also suggested an extramitochondrial site for the synthesis of cytochrome *c* in this tissue.

MATERIALS AND METHODS

Chemicals. L-[U- ^{14}C]Lysine (specific radioactivity 150mc/m-mole) was obtained from The Radiochemical Centre, Amersham, Bucks. Other chemicals were as described in the preceding paper (González-Cadauid & Campbell, 1967c).

Animals. Male Wistar albino rats (150 $\pm$ 10 g. body wt.) from a closed colony bred at this Institute were used after overnight starvation.

Procedure for injection of [^{14}C]lysine. Each rat was injected, under light ether anaesthesia, in the sublingual vein with 15 μC of [^{14}C]lysine dissolved in 0.25 ml. of 0.2 M-sodium phosphate buffer, pH 7.4 (0.2 M in Na^+). Groups of six rats were killed after 7.5, 15, 30 and 60 min.

Isolation and purification of cytochrome *c*. Details of the subcellular fractionation and the extraction, estimation and purification of cytochrome *c* were as given in the preceding paper (González-Cadauid & Campbell, 1967c). The following modifications were introduced to minimize the possible contamination of cytochrome *c* by adsorbed [^{14}C]lysine. Unlabelled L-lysine (0.1 ml. of a solution containing 10 mg./ml.) was added to each fraction containing cytochrome *c* immediately before each of the gel-filtration steps with Sephadex G-25. After completion of the chromatographic purification of cytochrome *c* and its spectrophotometric estimation, unlabelled lysine was again added and the protein subjected to conditions of high ionic strength and low pH to release any residual adsorbed [^{14}C]lysine. To effect this step it was necessary to add carrier horse heart cytochrome *c*. (The validity of using horse heart cytochrome *c* as a carrier was established, since both cytochromes *c* behaved similarly under the conditions of salt precipitation.)

Salt precipitation was carried out as follows. To the solution of pure rat liver cytochrome *c* was added unlabelled lysine (0.5 ml. of the same solution), 0.1 M- $\text{K}_3\text{Fe}(\text{CN})_6$ (0.1 ml.) and horse heart cytochrome *c* (1 ml. of a solution containing 1 mg./ml.) and the volume made up to 18 ml.

with 0.25 M- NH_4^+ buffer, pH 7.0. Then $(\text{NH}_4)_2\text{SO}_4$ was added to 80% saturation, and cytochrome *c* was precipitated with trichloroacetic acid at pH 3.5 and after 15 min. the precipitate collected by ultracentrifugation. The pellets were dissolved in 3.0 ml. of 0.02 M- NH_4^+ buffer, pH 7.0, containing 1 mg. of unlabelled lysine, and a minimum amount of $\text{Na}_2\text{S}_2\text{O}_4$ was added. The ionic strength of the solutions was lowered to 0.02 M- NH_4^+ by gel filtration on Sephadex G-25 and the cytochrome *c* concentrated on a column (1.0 cm. $\times$ 0.6 cm.) of Amberlite as in the preceding paper (González-Cadauid & Campbell, 1967c). Cytochrome *c* was eluted in 1.5 ml. of M-ammonium acetate and the concentration determined spectrophotometrically. The recovery of rat liver cytochrome *c* was estimated and was usually about 75%.

Where the amount of cytochrome *c* in the extract was very small carrier cytochrome *c* had to be added before chromatography. This applied to the material extracted with 0.15 M- NaCl from the heavy microsomal fraction. In such cases chromatographically pure rat liver cytochrome *c* was used as the carrier instead of horse heart cytochrome *c* in the above procedure. As a consequence of the addition of 'carrier' before chromatography the determination of the specific radioactivity of these cytochrome *c* preparations is less certain than in the former cases.

Preparation of total protein for radioactive determination. The protein from a sample of each of the subcellular fractions suspended in distilled water (before cytochrome *c* extraction) was precipitated with trichloroacetic acid (final concn. 10%, w/v). The pellets were washed as described by Campbell, Cooper & Hicks (1964) except that the ethanol contained potassium acetate (10%, w/v) to decrease protein losses (Munro & Downie, 1964; Steele, Okamura & Busch, 1964).

Radioactivity measurements. Cytochrome *c* solutions in M-ammonium acetate were plated directly on 7 cm.² disks and counted at infinite thinness in a gas-flow counter (Nuclear-Chicago Corp. model 4333) with low background (2.6 counts/min.). The efficiency of counting was 25%.

Total protein was dissolved by heating in concentrated formic acid (A.R. grade) for 30 min. at 90° (water bath). Samples were plated on 7 cm.² aluminium disks and counted at infinite thinness (less than 3 mg. of protein/disk) in a gas-flow counter (Nuclear-Chicago Corp. model 4330) with a background count of 12–15 counts/min. The efficiency of counting was 30%.

Radioactivity measurements are expressed as counts/min. obtained at 25% efficiency. Specific radioactivities were calculated from the amounts of original rat liver cytochrome *c* (spectrophotometric determinations corrected for the added carrier cytochrome *c*) and total protein (weighings) plated and expressed in counts/min./mg. of pure cytochrome *c* or total protein.

RESULTS

Incorporation of [^{14}C]lysine into total protein of subcellular fractions and mitochondrial subfractions. The incorporation of [^{14}C]lysine into the protein of the subcellular fractions followed the kinetics expected for liver as shown in Fig. 1. The specific radioactivities of a typical mitochondrial subfraction (no. 4) and of the nuclear fraction rose gradually to a plateau value at about 20 min., in

Fig. 1. Incorporation *in vivo* of $[^{14}\text{C}]$ lysine into total protein of rat liver subcellular fractions: $\square$, nuclear fraction; $\bullet$, mitochondrial subfraction 4; $\blacktriangle$, heavy microsomal fraction; $\circ$, standard microsomal fraction; $\triangle$, cell sap. Rats in groups of six were injected intravenously with $[^{14}\text{C}]$ lysine and killed at the stated times after injection. The livers were homogenized in 0.3 M-sucrose, the homogenates pooled and the fractions separated as described in the preceding paper (González-Cadavid & Campbell, 1967c).

Fig. 2. Incorporation *in vivo* of $[^{14}\text{C}]$ lysine into total protein and cytochrome *c* of rat liver mitochondrial subfractions: (a) mitochondrial subfraction 4; (b) comparison between the mitochondrial subfractions. Mitochondrial subfractions were obtained by sucrose-density-gradient centrifugation as described in the preceding paper (González-Cadavid & Campbell, 1967c). Cytochrome *c* was extracted from each subfraction brought to pH 4.0, first with water alone ($\circ$) and then with 0.15 M-NaCl ($\bullet$). It should be stressed that, in (b), for the sake of clarity no experimental points appear, the symbols being merely to identify the curves; only the average curves are plotted for subfractions 1-2 ($\triangle-\triangle$), 3 ($\bullet-\bullet$), 4 (—), 5 (---) and 6-7 ($\circ-\circ$). For other details see Fig. 1.

contrast with the two microsomal fractions, in which the specific radioactivity rose rapidly to a peak. The maximum specific radioactivity of the standard microsomal fraction was nearly six times that of the mitochondrial fraction. The peak of specific radioactivity of the heavy microsomal fraction was at about 20 min. and that of the standard microsomal fraction at 15 min. The specific radioactivity of the cell sap rose continuously to a value higher than that of the mitochondrial fraction.

The kinetics for incorporation into total protein were quite similar in all the mitochondrial subfractions, the specific radioactivity being higher in the subfractions from the top (6-7) and bottom (1-2) layers of the sucrose gradient, as shown in Fig. 2(b). These are the subfractions that are less rich in mitochondria. The lowest specific radioactivity (about 30% less than in subfraction 6-7) was that of subfraction 4, the subfraction that is least contaminated with fragments of endoplasmic reticulum (see González-Cadavid & Campbell, 1967c).

Incorporation of $[^{14}\text{C}]$ lysine into cytochrome c of mitochondrial subfractions and the standard microsomal fraction. The kinetics for incorporation into cytochrome *c* in mitochondrial subfraction 4 showed a nearly linear increase in specific radioactivity reaching a plateau value at about 30 min. that was about three times that for the total protein in this fraction (Fig. 2a). There was no significant difference between the specific radioactivities of the cytochrome *c* extracted with water or with 0.15 M-sodium chloride. The curves for the other subfractions are nearly identical with that for subfraction 4 (Fig. 2b). (Mitochondrial subfraction 1-2 is not included in the comparison since the amount of cytochrome *c* was too small for an accurate estimation of its specific radioactivity. For subfraction 8 only the determinations at 7.5 and 30 min. were completed and for the 0.15 M-sodium chloride-extracted protein the values were 110 and 190 counts/min./mg. respectively.) The kinetics for incorporation into the cytochrome *c* present in the standard microsomal fraction showed a rapid rise to a peak specific radioactivity that, for the 0.15 M-sodium chloride-extracted material, was about twice that for the total protein in the same fraction (Fig. 3). Both peaks occurred at the same time (about 15 min.) and there was a marked difference between the values for cytochrome *c* extracted with 0.15 M-sodium chloride and with water.

Incorporation of $[^{14}\text{C}]$ lysine into cytochrome c of other subcellular fractions. The incorporation into the cytochrome *c* in the heavy microsomal fraction is shown in Fig. 4. This indicates a rapid increase in the specific radioactivity of the water-extracted cytochrome *c* up to 7.5 min., the slope being

Fig. 3. Incorporation *in vivo* of $[^{14}\text{C}]$ lysine into total protein and cytochrome *c* of the standard microsomal fraction from rat liver. Cytochrome *c* was extracted from the fraction brought to pH 4.0, first with water alone ($\circ$) and then with 0.15M-NaCl ($\bullet$). Total protein values are represented by Δ . For other details see Fig. 1.

Fig. 4. Incorporation *in vivo* of $[^{14}\text{C}]$ lysine into total protein and cytochrome *c* of the heavy microsomal fraction from rat liver. Cytochrome *c* was extracted from the fraction brought to pH 4.0, first with water alone ($\circ$) and then with 0.15M-NaCl ($\bullet$). Total protein values are represented by Δ . For other details see Fig. 1.

much less steep thereafter. At 60 min. the specific radioactivity of cytochrome *c* is similar to that of the total protein in this fraction. Incorporation of $[^{14}\text{C}]$ lysine into the 0.15M-sodium chloride-extracted cytochrome *c* followed a different pattern, the specific radioactivity being much greater than that of the water-extracted protein. However, owing to the very small amounts of cytochrome *c* present in the 0.15M-sodium chloride-extractable pool of the heavy microsomal fraction (less than $0.7\mu\text{g./g.}$ of liver, and a total of $20\mu\text{g.}$ obtained from six livers) the accuracy of the determinations of specific radioactivity is low.

Fig. 5 shows the kinetics for the nuclear fraction. The extracted cytochrome *c* had a much higher specific radioactivity than that of the total protein of the fraction. Although the shapes of the curves for the two pools of cytochrome *c* are similar, the peak for the 0.15M-sodium chloride-extracted

Fig. 5. Incorporation *in vivo* of $[^{14}\text{C}]$ lysine into total protein and cytochrome *c* of the nuclear fraction from rat liver. Cytochrome *c* was extracted from the fraction brought to pH 4.0, first with water alone ($\circ$) and then with 0.15M-NaCl ($\bullet$). Total protein values are represented by Δ . For other details see Fig. 1.

Fig. 6. Incorporation *in vivo* of $[^{14}\text{C}]$ lysine into total protein (Δ) and cytochrome *c* ($\circ$) of the cell sap from rat liver. For details see Fig. 1.

Fig. 7. Comparison of the incorporation of $[^{14}\text{C}]$ lysine into cytochrome *c* of rat liver subcellular fractions: Cytochrome *c* was extracted from each fraction brought to pH 4.0, first with water alone (W) and then with 0.15M-NaCl (S). It should be stressed that, for the sake of clarity, no experimental points appear, the symbols being merely to identify the curves; only the curves already drawn independently in Figs. 2-6 are plotted for the nuclear fraction (---), mitochondrial subfraction 4 (—), heavy microsomal fraction ($\circ$ — $\circ$), standard microsomal fraction ($\bullet$ — $\bullet$) and the cell sap (Δ — Δ). For other details see Fig. 1.

cytochrome *c* occurred earlier than that for the water-extracted material. The incorporation into the cell sap is shown in Fig. 6. This indicates that the specific radioactivity of the water-extracted cytochrome *c* was much higher than that of the total protein in this fraction, although 60 min. after the injection of [^{14}C]lysine both had reached the same specific radioactivity.

*Comparison of the incorporation of [^{14}C]lysine into cytochrome *c* of mitochondrial subfractions and the other subcellular fractions.* In Fig. 7 the results for the kinetics of incorporation of [^{14}C]lysine into the cytochrome *c* of all the subcellular fractions are compared.

DISCUSSION

All the evidence from the very numerous studies on the structure of mitochondria and submitochondrial particles leads to the conclusion that cytochrome *c* is intimately associated with the structural components of this organelle. To suggest therefore that cytochrome *c* is not synthesized within the mitochondria is at first sight very surprising. If this should be the case several rather original problems in cellular physiology are raised. The failure to demonstrate the synthesis of cytochrome *c* in isolated preparations of mitochondria certainly cast doubt on the site of synthesis of this protein, but this evidence in itself was no more than suggestive. Indeed, the synthesis of cytochrome *c* by an isolated subcellular fraction has yet to be reported. The first necessity in this discussion is therefore to examine the kinetic evidence with a view to eliciting the initial site of synthesis of cytochrome *c* among the various subcellular fractions. It is argued that the most likely site is a component of the microsomal fraction. If the evidence is in favour of cytochrome *c* being synthesized by a component of the microsomal fraction that has no special link with the mitochondria then the problem arises as to how the protein is transferred from its site of synthesis to the mitochondria. Finally it has to be remembered that cytochrome *c* is a conjugated protein and that this in itself raises certain problems that must also be considered.

A comparison of the kinetics of the labelling with [^{14}C]lysine of cytochrome *c* and of the total protein of mitochondria shows that cytochrome *c* reaches its plateau value more slowly, but at a higher specific radioactivity than that of the total protein. Owing to the high lysine content of cytochrome *c* a comparison of the specific radioactivity with that of the total protein is probably of limited interest. The main point is the time at which the plateau is reached. The two times are clearly different and imply that the labelling of the cytochrome *c* pool of the mitochondria is slower than that of the total protein. This is in accord with the results of Beattie

et al. (1966) on the synthesis of the soluble protein of rat kidney mitochondria. Two interpretations of this result are possible: first, that cytochrome *c* is synthesized by the mitochondria at a lower rate than that of the total protein; secondly, that cytochrome *c* is synthesized outside the mitochondria and is transferred to this organelle.

Haldar *et al.* (1966) have shown that, when unlabelled amino acids are added to incubated mouse Krebs ascites-tumour cells to stop the incorporation of radioactive amino acids into protein, the specific radioactivity of cytochrome *c* continued to increase in a way resembling that of the cell-sap protein, whereas that of total mitochondrial protein and that in the whole homogenate remained stationary or even decreased. The results were interpreted as indicating an independent site for the synthesis of cytochrome *c* and mitochondrial protein and our findings are in complete agreement with this hypothesis.

When cytochrome *c* was extracted from mitochondria first with water and then with 0.15M-sodium chloride there was no significant difference between the specific radioactivities of the protein of the two extracts at any of the four times studied. The two pools of cytochrome *c* are likely to have been derived from different sites within the mitochondria. Thus irrespective of the origin of the mitochondrial cytochrome *c* it appears to be distributed at random throughout the mitochondrion as soon as it appears in a bound form.

In the preceding paper (González-Cadavid & Campbell, 1967c) it was shown that the heaviest and lightest mitochondrial subfractions were the most contaminated by fragments of the endoplasmic reticulum according to the analyses of glucose 6-phosphatase activity. It is therefore not surprising that the total protein of these subfractions too is more highly labelled than is that of the intermediate subfractions shown to be particularly rich in mitochondria (subfractions 4 and 5). If allowance is made for the microsomal contamination it is therefore probable that the rate of protein synthesis is independent of the density of the mitochondrial population. There was no significant difference between the specific radioactivities of cytochrome *c* from the various mitochondrial subfractions. In this case, owing to the marked difference in cytochrome *c* content, microsomal contamination would not significantly affect the results. Hence this finding indicates that the rate of appearance of newly synthesized cytochrome *c* in the mitochondria is independent of their density.

If we turn now to a consideration of the extra-mitochondrial components, the high specific radioactivity of cytochrome *c* in the standard microsomal fraction shortly after injection of [^{14}C]lysine

is the most striking observation. In contrast with the mitochondrial cytochrome *c* there is a marked difference in the specific radioactivities of the cytochrome *c* extracted with water and with 0.15M-sodium chloride, the latter having a much higher specific radioactivity than the former. The fact that the pool of newly synthesized cytochrome *c* is extracted only with 0.15M-sodium chloride can be interpreted as being due to its firm ionic binding to some morphological component of the microsomal fraction. If this component is ribosomal then, bearing in mind the basic properties of cytochrome *c*, it is reasonable to expect that the newly synthesized protein will be released by an ion-exchange displacement of cytochrome *c* from the phosphate groups of the ribosomal RNA by Na^+ ions (see Schwartz & Petermann, 1966). A similar phenomenon involving the phospholipid groups of a membrane component of the microsomal fraction is not excluded.

The fact that the kinetics for the 0.15M-sodium chloride- and water-extracted cytochrome *c* in the standard microsomal fraction resemble a precursor-product relationship suggests that the newly synthesized protein is first tightly bound and then more loosely bound. From this site in the microsomal fraction the cytochrome *c* may be transported to the mitochondria, where again it seems to be loosely bound.

The existence of some cross-contamination between the microsomal and mitochondrial fractions, together with the very great differences between the amounts of cytochrome *c* in the two pools, must be considered in any comparative analysis of the curves. A straight analysis of the ratio of the size of the two pools indicates that there is six times as much cytochrome *c* in the mitochondria as in the microsomal fraction. The real difference, however, is likely to be much greater since the microsomal fraction, on the basis of succinate-neotetrazolium reductase analysis, is contaminated with mitochondria, and it was calculated that this will contribute about half of the cytochrome *c* content of the microsomal fraction. In contrast, contamination of the mitochondrial fraction by fragments of the endoplasmic reticulum will only have a negligible effect on the cytochrome *c* content of this fraction. These considerations lead to the conclusion that the specific radioactivity of the mitochondrial cytochrome *c* will be unaffected by microsomal contamination, whereas the specific radioactivity of the cytochrome *c* in the microsomal fraction will be much decreased by mitochondrial contamination. The latter effect will be particularly prominent at early periods after the injection of [^{14}C]lysine, so that the trends revealed in the curves shown in Fig. 7 would, in reality, be much more dramatic.

For the standard microsomal fraction it should be noted that three times as much cytochrome *c* is extracted by water as by 0.15M-sodium chloride. This fact would help to explain why the peak of specific radioactivity of the former is lower than that of the latter.

An unexpected feature of the kinetics for the incorporation of [^{14}C]lysine into cytochrome *c* extractable with 0.15M-sodium chloride in the standard microsomal fraction was the plateau in specific radioactivity from 30 min., resembling the curve for the total protein in the same fraction. In the studies by Higashi & Peters (1963) on the biosynthesis of catalase in rat liver the same phenomenon was observed for the incorporation of [^{14}C]leucine into microsomal catalase. The relationship between the shapes of the curves for the microsomal and mitochondrial fractions is very similar for both catalase and cytochrome *c*. The interpretation of the shape of the curve for microsomal catalase was that there existed a pool of the enzyme in the microsomal fraction that underwent a slow turnover, and it seems probable that a similar explanation holds for the pool containing the newly synthesized cytochrome *c* in the present experiments. On this basis the final curve would be the result of two components: one, representing the newly synthesized cytochrome *c*, would be on a continuously downward path after the peak; the other, representing the cytochrome *c* transferred into a new pool of the same firmness of binding, would gradually increase in specific radioactivity. A similar phenomenon has been reported for the synthesis of glycoproteins in rat liver by Lawford & Schachter (1966), but in this case the synthesis of a specific protein was not studied.

The metabolic significance of the cytochrome *c* pool in the microsomal fractions having a slow turnover is a matter for speculation, but two explanations may be considered. The first is that cytochrome *c* is an essential component of the microsomal membranes and that these are undergoing synthesis. This would imply that cytochrome *c* plays a physiological role in the microsomal membranes, a possibility that is supported by the presence of an electron-transport chain in the microsomal membrane (Dallner, Siekevitz & Palade, 1966). So far no definite role for cytochrome *c* at this site has been claimed.

The second and more attractive explanation is that the cytochrome *c* that has been newly synthesized, and firmly bound to the ribosomes or membranes of the endoplasmic reticulum, would be transferred to a receptor unit in the microsome fraction, which also would contain a structural component of firmly bound cytochrome *c*. A striking feature of the kinetics for cytochrome *c* is that, whereas the specific radioactivity of the

0.15M-sodium chloride-extracted protein falls to a plateau from 30 min., as discussed above, that of the water-extracted protein continues to decrease and at 1 hr. has virtually the same value as that of the mitochondria. This is consistent with a sequence of events that involves, first, a change of the binding of cytochrome *c* within the microsomal fraction, so that the radioactivities of the water- and 0.15M-sodium chloride-extractable pools are equilibrated, and, secondly, with the transfer of the cytochrome *c* to the mitochondria. In this connexion Parsons, Williams, Thompson, Wilson & Chance (1967) have reported that the outer membrane of liver mitochondria resembles the smooth endoplasmic reticulum in lipid composition and in the presence of the cytochrome *b₅* electron-transport system. On the basis of these analyses and on morphological studies they have suggested that the outer mitochondrial membrane may be derived from the endoplasmic reticulum. If this hypothesis should prove to be correct a transfer of cytochrome *c* from the microsomal membranes to the mitochondria could be readily understood.

The idea of a transfer of cytochrome *c* bound to a structure such as the proposed receptor unit is at present speculative. An alternative explanation would be that the cytochrome *c* is released from the microsomal components in a soluble form. Lenaz & MacLennan (1966) have suggested that the outer membrane of the mitochondrion is permeable to cytochrome *c*. If this is so, an uptake of the protein in soluble form is possible and the idea is not excluded by our present results. However, the kinetics for cytochrome *c* in the cell sap suggest that contamination of this fraction with microsomal components is more likely. Higashi & Peters (1963) found a similar relationship between cell sap and microsomes for catalase.

With regard to the implications of cytochrome *c* being a conjugated haemoprotein, it is clear from our results that the complete cytochrome *c* molecule, and not merely the apoprotein, is assembled in the microsomal fraction. Thus, irrespective of the site of synthesis of the haem moiety, the apoprotein is linked to the prosthetic group in the morphological constituents of the microsomal fraction. Whether one or more constituents are involved in these processes is not at present known. It seems unlikely that the mechanism suggested by Haldar *et al.* (1966), implying that the cytochrome *c* apoprotein synthesized outside the mitochondrion is linked with the haem in the mitochondria, is correct. In this connexion it may be mentioned that it has not so far been possible to demonstrate the synthesis of cytochrome *c* by the isolated microsomal fraction (González-Cadavid & Campbell, 1967*b*) and it may be that this is due to a failure to conjugate the free apoprotein to the ferroporphyrin ring.

Though we favour the explanation of our results that implies an extramitochondrial site for the synthesis of cytochrome *c*, it is possible that they are due to a redistribution of cytochrome *c* during the process of cell disruption. According to this hypothesis the cytochrome *c* would be synthesized within the mitochondria with a subsequent redistribution of the newly synthesized protein and its selective attachment to some of the acidic groups of the standard microsomal fraction. Such an explanation would require that the newly synthesized cytochrome *c* is in a free (or a very weakly bound) form inside the mitochondria so that disruption of the liver cells in 0.3M-sucrose would be sufficient to break the linkage without affecting the rest of the mitochondrial pool of cytochrome *c*.

Apart from the work of Haldar *et al.* (1966) already discussed there are several other reports that indirectly support the extramitochondrial synthesis of cytochrome *c*. Kadenbach (1967) has shown that rat liver microsomes labelled *in vitro*, when incubated with mitochondria, are able to transfer radioactive proteins into various mitochondrial protein fractions but especially into the soluble proteins. Sherman, Stewart, Margoliash, Parker & Campbell (1966) have demonstrated that the structural gene for the synthesis of cytochrome *c* in yeast is outside the mitochondria. Sinclair & Stevens (1966) showed that the amount of DNA present in the mitochondria is sufficient only to provide genetic information for the synthesis of a limited number of mitochondrial proteins. This may be taken to imply that non-structural mitochondrial proteins are synthesized elsewhere.

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