The Significance of Cytochrome *c* Redistribution during the Subcellular Fractionation of Rat Liver

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1. The redistribution of mitochondrial cytochrome *c* during homogenization and subcellular fractionation of the liver was studied. Chromatographically homogeneous ^{14}C -labelled cytochrome *c* was added in different amounts to liver suspensions immediately before homogenization and the adsorption of radioactivity was determined in cytochrome *c* fractions extracted at pH 4.0, first with water and then with 0.15M-sodium chloride. 2. The soluble cytochrome *c* remaining in the cell sap after subcellular fractionation was 7% of the calculated amount of cytochrome *c* passing through a soluble form during the whole process. The total amount of cytochrome *c* released in a soluble form and subsequently redistributed was 25–30% of the total liver cytochrome *c*. 3. In the standard microsomal fraction the cytochrome *c* extracted with water originated entirely from redistribution whereas that extracted with 0.15M-sodium chloride was 80% endogenous. In the mitochondrial fraction both cytochrome *c* pools were truly endogenous, so that practically none of the mitochondrial cytochrome *c* released to the soluble cell sap was readsorbed by the mitochondria. 4. These results support our former hypothesis that the cytochrome *c* extracted with 0.15M-sodium chloride at pH 4.0 from the standard microsomes represents the cytochrome *c* newly synthesized *in situ*, since it does not originate from redistribution. However, the microsomal pool extracted with water cannot be an intermediate in the postulated transfer of cytochrome *c* from the microsomal particles to the mitochondria, since this pool arises from redistribution of mitochondrial cytochrome *c*.

That the amount of cytochrome *c* associated with subcellular fractions from disrupted rat liver depends on the homogenizing medium was first shown by Schneider, Claude & Hogeboom (1948) and Schneider & Hogeboom (1950). They postulated that all the cytochrome *c* of the cell was contained within the mitochondria and that during cell disruption and fractionation some of it was redistributed to other organelles. Beinert (1951), using radioactively labelled cytochrome *c*, confirmed these ideas by demonstrating that the protein was released from the mitochondria in a soluble form during cell disruption and that it was subsequently redistributed.

González-Cadavid & Campbell (1967*a*) studied the subcellular distribution of cytochrome *c* in rat liver by using specifically designed procedures for subcellular fractionation (in 0.3M-sucrose) and for

the extraction and purification of cytochrome *c*. Although these results for the distribution of cytochrome *c* differed in some respects from those previously reported, they confirmed that about 40% of the total cytochrome *c* was extramitochondrial. Of this amount only half could be accounted for by mitochondrial contamination as judged by succinate-neotetrazolium assays. In further work (González-Cadavid & Campbell, 1967*b*) it was shown that a minor pool of cytochrome *c*, extractable from the 'standard' microsomal fraction at pH 4.0 with 0.15M-sodium chloride, contained the recently synthesized cytochrome *c*. The possible significance of the redistribution of cytochrome *c* on the results obtained in both papers was discussed. The present paper describes the results of experiments with two main objectives in view.

First, we wished to determine the proportion of cytochrome *c* that is truly endogenous to the different organelles contained in the subcellular fractions, with particular reference to the microsomal pool containing the recently synthesized cytochrome *c* (isolated at pH 4.0 with 0.15M-sodium chloride). Secondly, we wished to determine the proportion of cytochrome *c* in the cell sap

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that is truly soluble and its relation to a hypothetical soluble pool pre-existing in the cell before disruption.

The experiments of Beinert (1951) do not provide the answer to these problems and are in any case subject to criticism on the grounds of the purity of the cytochrome *c* and the procedures for subcellular fractionation and extraction of cytochrome *c* (see González-Cadavid & Campbell, 1967b). In the present work chromatographically pure cytochrome *c* was used as a 'marker' and improved methods were used for subcellular fractionation and extraction of cytochrome *c*, as described by González-Cadavid & Campbell (1967a).

MATERIALS AND METHODS

Chemicals. U-¹⁴C-labelled *Chlorella* protein hydrolysate (specific radioactivity 0.6 mc/mg.) and L-[U-¹⁴C]lysine (specific radioactivity 180 mc/m-mole) were obtained from The Radiochemical Centre, Amersham, Bucks. Other chemicals were as described by González-Cadavid & Campbell (1967a).

Animals. Male Wistar albino rats from a closed colony bred at this Institute, 150 ± 10 g. body wt., were used after overnight starvation. For the preparation of cytochrome *c* labelled *in vitro*, litters of non-starved 5-day-old rats were used.

Nomenclature of cytochrome *c* extracts. The following abbreviations are used (González-Cadavid & Campbell, 1967a): cytochrome *c* (W), cytochrome *c* extracted from tissue homogenates in distilled water, brought to pH 4.0; cytochrome *c* (NaCl), cytochrome *c* extracted from the residues, after extraction in distilled water, with 0.15 M-NaCl.

Preparation of pure ¹⁴C-labelled cytochrome *c*. Radioactive cytochrome *c* was prepared from rat liver by two different procedures.

Expt. 1. Each of three rats were injected intraperitoneally with 25 μC of ¹⁴C-labelled protein hydrolysate dissolved in 1.5 ml. of 0.2 M-sodium phosphate buffer (0.2 M in Na⁺), pH 7.4. They were killed at 4 hr. and the cytochrome *c* was extracted from the liver and purified as described by González-Cadavid & Campbell (1967a,b) but with omission of the treatment with (NH₄)₂SO₄ and trichloroacetic acid, which may cause partial denaturation (see, e.g., Margoliash, 1954).

Expt. 2. Cytochrome *c* with a higher specific radioactivity was obtained by the incubation of liver slices. Slices were obtained from the livers of 5-day-old rats and were incubated for a period of 4 hr. under the conditions of Peters & Anfinsen (1950) in the presence of [¹⁴C]lysine with the addition of glucose (0.02 M) and an amino acid mixture (0.25 mM each) lacking lysine. Cytochrome *c* was extracted and purified as in Expt. 1. The chromatographic properties of the cytochrome *c* labelled *in vitro* were identical with those of the cytochrome *c* labelled *in vivo*.

Specific radioactivity of ¹⁴C-labelled cytochrome *c*. This was determined as described by González-Cadavid & Campbell (1967b). For preparation 1 the specific radioactivity was 314 counts/min./mg. Preparation 2 was divided into two samples. The specific radioactivity of one (A) was determined without further treatment and was 20890 counts/min./mg.; the second sample (B) was first treated with

(NH₄)₂SO₄ and trichloroacetic acid, which decreased the specific radioactivity to 19870 counts/min./mg., a fall of 4.0% probably attributable to contamination of sample A with free [¹⁴C]lysine. In the calculation below the specific radioactivity of cytochrome *c* preparation 2 is therefore corrected to the lower value. Before use preparation 2 was diluted with chromatographically-pure unlabelled rat liver cytochrome *c* to give a final specific radioactivity of 316 counts/min./mg.

Redistribution experiments. Expt. 1. A solution (3.6 ml.) containing 1.46 mg. of ¹⁴C-labelled cytochrome *c* (preparation 1) in 0.3 M-sucrose-0.02 M-ammonium acetate was divided into six portions. Each portion was added to the mince of a liver from one 150 g. rat, suspended in 15 ml. of 0.3 M-sucrose. The mince was homogenized as described by González-Cadavid & Campbell (1967a). The procedure was repeated with six rat livers (total weight 32.7 g., total amount of cytochrome *c* used 1.46 mg.) and the homogenates were pooled. Subcellular fractionation was performed as described by González-Cadavid & Campbell (1967a) except that the cell sap was further treated as follows. The cell sap was centrifuged for 16 hr. at 55000g, which resulted in a gelatinous red pellet. During decantation of the supernatant a minor loose portion of the pellet was removed. The supernatant (about 330 ml.) was then filtered successively through 1.2 μ- and 0.22 μ-pore-size Millipore filters (47 mm. diam., used with pre-filters) at an N₂ pressure of 5 lb./in.². The filtrate was designated 'cell sap (soluble)'. The filters were chopped with scissors and pooled with the gelatinous pellets. This fraction was designated 'cell sap (residue)'.

Cytochrome *c* was extracted from the subcellular fractions and partially purified by the initial steps described by González-Cadavid & Campbell (1967a). The chromatography on the 20 cm. Amberlite IRC-50 column (at pH 7.0) was omitted, as was the previous gel-filtration on Sephadex G-25, since the purification required was merely to eliminate impurities that could interfere with the determination of cytochrome *c*. The extracts of the mitochondrial and heavy-microsomal fraction were pooled and processed as a single fraction. For the cell sap (residue) the volumes of the 5 cm. columns used were 3.4 ml. (pH 7.0) and 2.0 ml. (pH 9.0). The final eluates from the columns were obtained in M-ammonium acetate. When portions were taken for radioactivity determination suitable corrections for self-absorption were applied.

Expt. 2. The same procedure was employed as in Expt. 1, except that ¹⁴C-labelled cytochrome *c* sample 2 was used as marker. The total amount of liver was 29 g. and the total amount of preparation 2 cytochrome *c* was 11.22 mg.

Determination of the recovery of cytochrome *c* added to the cell sap. To determine whether the Millipore filters used in the above procedure adsorbed soluble cytochrome *c*, horse heart cytochrome *c* in 0.3 M-sucrose was passed through such filters. A quantitative recovery was obtained.

The recovery of added cytochrome *c* from the cell sap (soluble) was determined as follows. The sample was divided into two equal parts, to one of which a known amount of horse heart cytochrome *c* was added. Cytochrome *c* was extracted from each and the recovery determined.

RESULTS

The results obtained in both experiments are shown in Tables 1-3 and a schematic interpretation is given in Scheme 1. Before these results are

Table 1. Amount of bound cytochrome *c* truly endogenous in each subcellular fraction from rat liver

¹⁴C-Labelled cytochrome *c* (specific radioactivity 315 counts/min./mg.) was added to 32.7 g. (Expt. 1) and 29.0 g. (Expt. 2) of liver mince in 0.3 M-sucrose immediately before homogenization. The amounts added were 1.46 mg. and 11.22 mg. respectively. Subcellular fractionation and cytochrome *c* extractions, purification and determination were performed as described in the text. For the calculation of results appearing in column (C), the quotient between values (A)/(B) was subtracted from the amount of total cytochrome *c* in the fraction (initial+added) and divided by the liver weight.

Cytochrome <i>c</i> fractions extracted at pH 4.0	Cytochrome <i>c</i>					
	Expt. 1			Expt. 2		
	(A) Total radio-activity present (counts/min.)	(B) Sp. radio-activity (counts/min./mg.)	(C) Amount endogenous (bound) (μg./g. of liver)	(A) Total radio-activity present (counts/min.)	(B) Sp. radio-activity (counts/min./mg.)	(C) Amount endogenous (bound) (μg./g. of liver)
(a) With water						
Nuclear	83	104	2.7	1046	258	15.6
Mitochondria + heavy microsomes	87	31	62.3	438	132	62.0
Standard microsomes	73	149	0	950	298	0
Cell sap (pellet + residue)	18	119	0	135	276	0.7
Cell sap (soluble)	24	117	—	274	290	—
Sum of fractions (a)	285	—	65.0	2843	—	78.3
(b) With 0.15 M-NaCl						
Nuclear	3.2	33	2.1	49	174	3.8
Mitochondria + heavy microsomes	6.8	11	18.0	50	83	14.8
Standard microsomes	4.8	35	3.0	95	213	4.1
Cell sap (pellet + residue)	0.9	133	0	13	236	0.3
Cell sap (soluble)	2.9	85	0.3	8	174	0.6
Sum of fractions (b)	19	—	24.3	215	—	23.6
(c) Sum of fractions (a) + (b)	304	—	88.4	3058	—	101.9

considered certain basic assumptions and requirements for the techniques and calculations employed must be made clear.

Theoretical requirements. They are as follows. (a) The marker cytochrome *c* is added immediately before cell disruption. It must be assumed that it will come instantaneously into equilibrium with the mitochondrial cytochrome *c* released in a soluble form during the homogenization and subcellular fractionation. (b) The marker cytochrome *c* has to be not only pure but also undenatured to avoid heterogeneous behaviour of the soluble cytochrome *c* pool. (c) The amount of marker cytochrome *c* added should not influence the proportion of cytochrome *c* that is released from the mitochondria. (d) The specific radioactivity of the marker cytochrome *c* should be reasonably constant throughout the homogenization and fractionation. (e) Cell disruption and fractionation must be carried out by a reproducible procedure. (f) The cell sap has to be free from particulate material so that the value for the specific radioactivity of the soluble cytochrome *c* used in the calculations is not subject to errors due to the presence of bound cytochrome *c*. (g) The

cytochrome *c* isolated from the subcellular fractions has to be purified sufficiently to eliminate impurities that could interfere with the determination of cytochrome *c*.

The procedures employed in the present investigations clearly fulfilled all these conditions except (c) and (d), which require further consideration.

With regard to (d), a variable value for the specific radioactivity of the soluble pool of cytochrome *c* would cause a serious error in the determination of the amount of soluble cytochrome *c* adsorbed by each fraction and hence in the calculated amount of cytochrome *c* redistribution. Cell disruption is never an instantaneous process and in the present procedure three separate homogenizations were involved. Damage to the mitochondria may well progressively increase, with a concomitant release of cytochrome *c*. At the same time part of the soluble pool of cytochrome *c* will be continuously adsorbed by the subcellular components released during homogenization. It is clear that, if the amount of marker cytochrome *c* added is small with respect to the amount of cytochrome *c* released from the mitochondria, then the specific radioactivity of the

Table 2. *Proportion of bound cytochrome c truly endogenous in each subcellular fraction from rat liver*

The ratios between the specific radioactivity of the 'marker' cytochrome *c* and that of the cytochrome *c* in the soluble cell sap were 2.68 in Expt. 1 and 1.09 in Expt. 2. The amounts of total soluble cytochrome *c* (initial + added) at zero time calculated from this isotope-dilution factor were 3.91 mg. and 12.23 mg. respectively. The proportions of these values contributed by the liver homogenates (i.e. after subtraction of the contribution of the marker cytochrome *c*) were 62.6 and 8.2%, respectively. These values multiplied by the amount of soluble cytochrome *c* present or adsorbed in each fraction and divided by the liver weight gave the results shown in column (A). For other details see Table 1.

	Cytochrome <i>c</i>					
	Expt. 1			Expt. 2		
	(A) Adsorbed from soluble pool ($\mu\text{g./g. of}$ liver)	(B) Total (endogenous + adsorbed) ($\mu\text{g./g. of liver}$)	(C) 100 $\times$ Endogenous Total (%)	(A) Adsorbed from soluble pool ($\mu\text{g./g. of}$ liver)	(B) Total (endogenous + adsorbed) ($\mu\text{g./g. of liver}$)	(C) 100 $\times$ Endogenous Total (%)
Cytochrome <i>c</i> fractions extracted at pH 4.0						
(a) With water						
Nuclear	13.6	16.3	17	10.3	25.9	60
Mitochondria + heavy microsomes	14.3	76.6	81	4.3	66.3	94
Standard microsomes	11.9	11.9	0	9.4	9.4	0
Cell sap (pellet + residue)	2.9	2.9	0	1.3	2.0	35
Cell sap (soluble)	3.9	3.9	—	2.7	2.7	—
Sum of fractions (a)	46.6	111.6	58	28.0	106.3	74
(b) With 0.15 M-NaCl						
Nuclear	0.5	2.6	81	0.5	4.3	88
Mitochondria + heavy microsomes	1.1	19.1	94	0.5	15.3	97
Standard microsomes	0.8	3.8	79	0.9	5.0	82
Cell sap (pellet + residue)	0.2	0.2	0	0.1	0.4	75
Cell sap (soluble)	0.4	0.7	43	0	0.6	100
Sum of fractions (b)	3.0	26.4	89	2.0	25.6	92
(c) Sum of fractions (a) + (b)	49.6	138.0	64	30.0	131.9	77
						28.2 58.9 10.9 0.9 2.5 101.4 2.5 18.4 3.3 0.3 24.8 126.2

* Values obtained before chromatography, taken from González-Cadauid & Campbell (1967a). Values for cell sap are those from the present paper.

Table 3. Amounts of cytochrome *c* redistributed during subcellular fractionation of rat liver

Values in line 6 were obtained from the addition of individual values appearing in Table 2, column (B), and refer to cytochrome *c* originally present in the tissue. Results in line 8 were calculated by multiplying values from line 6 by the liver weight and adding the amount of marker cytochrome *c* used. Values in line 11 differ from those in line 6 in being calculated by subtracting the contribution of the marker cytochrome *c* from the values (see Table 2) of total soluble cytochrome *c* at zero time and then dividing by the liver weight. For other details see Tables 1 and 2.

Cytochrome <i>c</i>	Units	Expt. 1	Expt. 2
1. Before addition of 'marker' (sum of fractions)	mg.	4.51	3.83
2. After addition of 'marker'	mg.	5.97	15.05
3. Recovered after fractionation (amount)	%	89.5	89.2
4. Total radioactivity, added*	counts/min.	459	3549
5. Total radioactivity, recovered*	%	66.2	86.2
6. Amount redistributed, from adsorption of label	μg./g. of liver	49.6	30.0
7. Proportion redistributed, of total in liver	%	36	23
8. Total soluble (initial + added), at zero time	mg.	3.09	12.09
9. Total soluble (initial + added), in cell sap at end of fractionation	mg.	0.21	0.94
10. Soluble pool, proportion remaining ($100 \times \frac{\text{line 9}}{\text{line 8}}$)	%	6.8	7.8
11. Amount redistributed, from dilution of label	μg./g. of liver	74.9	34.8

* Suitable corrections to compensate for any adsorbed radioactive lysine were only applied in Expt. 2, as described in the text.

Scheme 1. Representation of the postulated steps of transfer and redistribution of cytochrome *c* within rat liver subcellular fractions. The transfer pathways that would be operative in the biosynthesis of cytochrome *c* *in vitro* are indicated by —→. The redistribution effects occurring during cell disruption and subcellular fractionation are shown by ----→.

soluble cytochrome *c* will progressively decrease from the start of homogenization. To avoid this complication it is best to maintain a constant specific radioactivity of the soluble pool by adding a large excess of marker cytochrome *c*. To test this hypothesis markedly different amounts of labelled cytochrome *c* were added in the two experiments. In Expt. 1 the amount of marker cytochrome represented 60% of the endogenous soluble pool and the specific radioactivity of the soluble pool

after addition of marker fell from an initial value of 314 to a final value of 177 counts/min./mg. of cytochrome *c*. In Expt. 2 the comparable values were an 11-fold excess of marker cytochrome *c* and initial and final specific-radioactivity values of 316 and 290 counts/min./mg. of cytochrome *c* respectively (see Table 2).

With regard to requirement (c), it is assumed that cytochrome *c* is bound to the mitochondria by ionic linkage (e.g. see Jacobs & Sanadi, 1960; Margoliash & Shejter, 1966) and that the proportion of cytochrome *c* 'eluted' from damaged mitochondria will not depend on the concentration of cytochrome *c* in the soluble medium (in analogy with that of a cation-exchange process).

Cell-sap cytochrome *c*. Table 2 shows that about two-thirds of the cytochrome *c* in the liver cell sap is in a truly soluble form. Of the cytochrome *c* in the cell sap (soluble) virtually all was extracted by adjustment of the pH to 4.0.

In both experiments the addition of marker cytochrome *c* before homogenization led to an increase in the final amount of cytochrome *c* in the cell sap (soluble) after subcellular fractionation, as shown in Table 3 (line 9). The size of this pool is about 7% of the calculated soluble cytochrome *c* at zero time irrespective of its amount and concentration (Table 3, line 10). As shown below, this ratio seems to be maintained even in the absence of added marker cytochrome *c*.

The recovery of the added cytochrome *c* both in terms of weight and in terms of radioactivity was reasonable for Expt. 2. For Expt. 1 the amount recovered was reasonable but the recovery of

radioactivity was low (see Table 3, lines 3 and 5). The most likely fraction to be implicated in such losses would be the cell sap, containing high soluble-protein and cation concentrations that might well interfere with cytochrome *c* isolation. In addition, the specific radioactivity of the cytochrome *c* was highest in this fraction. In the recovery experiments performed with the cell sap (soluble) only 75% of the 3mg. of added cytochrome *c* was recovered in the water extraction and a further 3% in the 0.15M-sodium chloride extraction. This amounts to 22% (0.7 mg.) of the added cytochrome *c* lost in half of the usual volume of the cell sap (soluble).

The cytochrome *c* (W) specific radioactivities of the cell sap (soluble) were lower than those of the added 'markers' owing to dilution by the redistributed cytochrome *c* (Table 1). In Expt. 2 the dilution factor was virtually that to be expected from the existence of a pool of redistributed cytochrome *c* of the size calculated from the radioactivity adsorbed. In Expt. 1 this was not so and was due to the variation of the specific radioactivity of this pool as discussed above.

Proportion of bound cytochrome c that is truly endogenous in each particulate subcellular fraction. By this term we mean the proportion of the total cytochrome *c* bound to each particulate fraction that does not arise from redistribution of a soluble pool. In Table 2 it is shown that in both experiments none of the cytochrome *c* (W) in the standard microsomal fraction was truly endogenous. In contrast about 80% of the cytochrome *c* (NaCl) in the same fraction was endogenous. The specific radioactivity of the microsomal cytochrome *c* (W) in Expt. 1 was higher than that of the cytochrome *c* in the cell sap (soluble) (see Table 1). This is a consequence of the variation of the specific radioactivity of the soluble cytochrome *c* and indicates that most of the microsomal cytochrome *c* (W) arises immediately from the soluble pool, before the further release of mitochondrial cytochrome *c* dilutes the radioactivity of the soluble cytochrome *c*. Since the specific radioactivity of the soluble cytochrome *c* in Expt. 2 was nearly constant this difference between the specific radioactivities of the cell-sap and microsomal cytochrome *c* was negligible.

It is also important to consider that, if some added cytochrome *c* has become adsorbed on the surface of the liver cells in the mince, it may well appear as radioactivity in the cytomembrane (microsomal) fraction. However, the fragments of cell membrane usually present in the microsomal fraction are only a minimum proportion of the original cell membranes, which are concentrated in the 6000g-min. nuclear fraction, whereas the total amount of radioactivity adsorbed in the microsomal fraction is nearly as high as that present in the nuclear fraction (Table 1).

This fact indicates that, though the adsorption of labelled cytochrome *c* before homogenization might contribute to an increase in the specific radioactivity of the microsomal fraction, this effect is not very significant.

In contrast with the cytochrome *c* (W) from the standard microsomes, practically all the cytochrome *c* [both (W) and (NaCl)] found in the mitochondrial plus heavy-microsomal fraction is truly endogenous (Table 2). In the nuclear fraction the endogenous cytochrome *c* (W) is a much smaller proportion of the total but about 85% of the cytochrome *c* (NaCl) is endogenous.

There is an appreciable difference between the two experiments with respect to cytochrome *c* (W) in the nuclear fraction and this is probably due to the change of specific radioactivity of the soluble cytochrome *c* as explained for the microsomal fraction. Another cause of the variation might be that in Expt. 1 fewer mitochondria were trapped in the nuclear fraction (Table 2).

From Table 2 it was calculated that only about 10% of the total cytochrome *c* (NaCl) of the particulate subcellular fractions arises from the soluble pool, in contrast with cytochrome *c* (W) where 27% was redistributed in Expt. 2 and 42% in Expt. 1.

Amount of cytochrome c redistributed during subcellular fractionation of rat liver. This is the total amount of cytochrome *c* in the liver cell released from the mitochondria to a soluble form that is later bound by particulate subcellular components. The two methods of calculating this amount gave satisfactory agreement in Expt. 2 but different results (see Table 3, lines 6 and 11) in Expt. 1. The method based on the dilution of the soluble specific radioactivity is subject to more error than the procedure based on the radioactivity adsorbed by the particulate components. This is because the lack of constancy in the specific radioactivity of the soluble cytochrome *c* will affect the first method of calculation more than the second. For this reason the preferred values are obtained from the radioactivity adsorbed (Table 3, lines 6 and 7) and show that 23–30% of the total liver cytochrome *c* is involved in redistribution.

DISCUSSION

A comparison of the results in the two experiments clearly shows that for a valid interpretation the specific radioactivity of the soluble cytochrome *c* must be kept reasonably constant throughout the process of cell disruption. This was substantially achieved in Expt. 2. Even so, the conclusion on the significance of the redistribution of cytochrome *c* can only be applied strictly to a subcellular fractionation carried out exactly under the conditions

applied in the present study. We believe, however, that the results are of general application in all systems involving cell disruption and subcellular fractionation.

The components in the cytoplasm that adsorb the cytochrome *c* may be likened to an ion-exchange resin. The amount of the soluble cytochrome *c* adsorbed is proportional to its concentration over at least a tenfold range. Thus about 7% of the cytochrome *c* remains soluble irrespective of the amount added. In the absence of added cytochrome *c* the small amount of truly soluble protein in the cell sap may be considered to represent the residue of a much larger pool released from the mitochondria. It is not possible at present to decide whether the cell contains any soluble cytochrome *c* before its disruption.

The amount of cytochrome *c* present in liver subcellular fractions other than the mitochondria or the heavy-microsomal fraction (probably containing light mitochondria) represents about 38% of the total. About 65% of this 'extramitochondrial' cytochrome *c* is now shown to be of mitochondrial origin, leaving 35% as possibly truly endogenous to the 'extramitochondrial' fractions. Previous assays on succinate-neotetrazolium reductase (González-Cadavid & Campbell, 1967*a*) indicated that the latter fractions were contaminated with intact or fragmented mitochondria, so that only a proportion of this apparent extramitochondrial cytochrome *c* is of non-mitochondrial origin.

The released mitochondrial cytochrome *c* is taken up in different proportions by the binding sites involved in the extractions at pH 4.0 with water or with iso-osmotic sodium chloride solution. Of the total cytochrome *c* bound to the particles that is extractable with water, 65% is exogenous to the fraction and of that extracted with sodium chloride solution only 20% is exogenous.

Only a very small proportion (about 5% in Expt. 2) of the soluble cytochrome *c* is readsorbed by the mitochondria, in close correlation with the 10% found by Beinert (1951). In contrast the standard microsomes are avid for soluble cytochrome *c* and all of that which is extracted from this fraction with water seems to be of mitochondrial origin. However, most of the microsomal cytochrome *c* (NaCl) is endogenous.

In view of these new results a more accurate interpretation of our former time-radioactivity curves for the synthesis of cytochrome *c* in the intact animal (González-Cadavid & Campbell, 1967*b*) can be made. First, it is clear that our former suggestion that the microsomal cytochrome *c* (NaCl) represents the cytochrome *c* newly synthesized *in situ* holds true, for this fraction is shown to be in the main endogenous. However, our suggestion that the cytochrome *c* on transfer from the microsomal

particles to the mitochondria passes through an easily extractable form is not necessarily correct, for it is now realized that this cytochrome *c* arises from the mitochondria (see Scheme 1). The fact that in our former experiments microsomal cytochrome *c* (W) was much more radioactive than mitochondrial cytochrome *c* can be explained in two ways. Either some of the newly synthesized cytochrome *c* enters the soluble pool and then becomes by-passed in the microsomal cytochrome *c* (W) pool or the newly synthesized protein entering the mitochondria is preferentially released when the organelle is damaged during cell disruption. This would imply either that the recently synthesized cytochrome *c* is more easily extractable or that it is concentrated in a particularly fragile fraction of the mitochondrial population.

This last hypothesis is rendered less likely by the work of Lusena & Depocas (1966). These authors have shown that, when the mitochondrial fraction of rat liver was separated into five subfractions by differential centrifugation, the heavier subfractions were more fragile than the lighter ones, as assessed by freezing at -5° followed by thawing at 37° , or exposure to hyperosmotic sucrose solutions. However, no correlation could be found between the different fragilities *in vitro* and the pattern *in vivo* of labelling with sodium [^{14}C]acetate of the mitochondrial subfractions, suggesting that this differential fragility is not related with the 'age' of the mitochondrial population. Therefore it is unlikely that the new cytochrome *c* recently transferred to the mitochondria *in vivo* would be particularly concentrated in more fragile mitochondria.

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