ORIGIN OF MITOCHONDRIAL ENZYMES (II). THE SUBCELLULAR DISTRIBUTION OF CYTOCHROME *c* IN RAT LIVER TISSUE

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1. Introduction

Cadavid and Campbell [1], using radioactive lysine as a precursor, have reported that cytochrome *c* is synthesized by the endoplasmic reticulum. This laboratory, using δ-[^{3}H]aminolevulinic acid as a precursor, has confirmed [2,3] Cadavid and Campbell's findings and shown that final assembly of the cytochrome *c* molecule must occur in the endoplasmic reticulum. These results verify, with respect to a specific mitochondrial protein, previous findings of an apparent inability of mitochondria to synthesize all their constituent proteins [4–6]. In the course of studying its synthesis, we have also determined the intracellular distribution of cytochrome *c*. In such studies we have found that more than 90% of the total cytochrome *c* of the liver cell is localized in the mitochondria; about 4% is found in the microsomes and the remainder is divided approximately equally between the nuclei, premicrosomes and the cell sap.

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2. Experimental procedures

The techniques of liver-tissue derivation and fractionation and the procedures of extraction, purification and determination of cytochrome *c* have been described previously [3]. Six animals were used per experiment to enable accurate determination of the cytochrome *c* in the extra-mitochondrial fractions. The cytochrome *c* content of the subcellular fractions was determined at room temperature; that of whole liver homogenates was determined as the sum of the contents of the subcellular fractions. Other experiments, to be presented in detail elsewhere, establish that cytochrome *c* extraction from all subcellular fractions is quantitative. The yields of the various cell fractions and the extent of cross-contamination were estimated by determination of the following marker substances: nuclei, DNA [7]; mitochondria, cytochromes $a + a_3$ [8]; microsomes, α-D-glucose 6-phosphate phosphohydrolase [9]. By such means it was established that the nuclei were free of mitochondria and microsomes and that the microsomes were free of intact mitochondria. However, there was demonstrable contamination of the mitochondria by microsomes. Protein concentrations were determined by the biuret procedure [10].

Table 1
The content and distribution of cytochrome *c* in rat liver and isolated subcellular fractions.

	Cytochrome *c* content (μg/mg protein)					Percent distribution			
Reference	*	[11]	[12]	[13]	[14]	*	[11]		
							a	*b*	*c*
Sum of fractions	0.66	0.68				100.0			
Nuclei	0.40	1.07	0.35	0.12		1.9 (1.3–2.6)	24.4	17.0	2.0
Mitochondria	2.59	1.76				91.2 (88–95)	57.2	71.4	91.1
Premicrosomes	0.06	0.54				1.2 (0.5–2.0)	5.2	2.4	0.6
Microsomes	0.08	0.35			0.13	3.7 (1.3–5.3)	10.6	6.4	3.1
Cell sap	0.07	0.05				1.7 (1.3–2.8)	2.7	2.7	2.7

* Results of six separate experiments of this laboratory.
a Percent distribution reported by Cadavid and Campbell [11].
b Adjustment of results of column *a* for content of intact mitochondria in extra-mitochondrial particulate fractions [11].
c Adjustment of results of column *a* for cytochrome *c* (W) content of extra-mitochondrial particulate fractions [11].

3. Results and discussion

Table 1 presents the results of six separate experiments of this laboratory wherein the content of cytochrome *c* in liver tissue and its subcellular localization were determined. These results have been corrected for the contamination of mitochondria by microsomes; no other corrections were necessary. For purposes of comparison the findings of Cadavid and Campbell [11] and of other investigators are also presented. Our results are in good agreement with those of Cadavid and Campbell [11] in regard to the μg of cytochrome *c*/mg protein in whole homogenates. However, it is apparent that the cytochrome *c* content of various cell fractions found by Cadavid and Campbell [11] is in striking disagreement, not only with the results of this laboratory, but also with the results of other investigators who had previously determined the cytochrome *c* content of specific extra-mitochondrial fractions.

Table 1 presents as well the average percentage distribution of the total cytochrome *c* in the various cell fractions. We have found that liver mitochondria contain a minimum of 91% of the total cytochrome *c*; the microsomes contain about 4%; the remaining 5% is distributed in roughly equal amounts in each of the other cell fractions. Table 1 contains also the comparable results from the work of Cadavid and Campbell [11]. With the exception of the cytochrome *c* content of the cell sap, their uncorrected values (column *a*, table 1) for the percentages of the cytochrome *c* in various cell fractions are widely disparate from the results of this laboratory. This disparity is diminished somewhat when the extra-mitochondrial contents of cytochrome *c* are corrected for the content of intact mitochondria in those fractions (column *b*, table 1). However, after such a correction, the percentage of the total cytochrome *c* in the isolated mitochondria found by Cadavid and Campbell [11] is still 20% less than we found in our experiments (table 1).

In their work, Cadavid and Campbell [11] found that substantial portions of the cytochrome *c* of each of the particulate cell fractions could be extracted therefrom with distilled water at pH 4.0, prior to any extraction with salts. They concluded that the water-extractable [cytochrome *c* (W)] and salt-extractable [cytochrome *c* (NaCl)] forms constituted two distinct pools of that protein. We have believed it more likely that a large part of the cytochrome *c* (W) of the extramitochondrial fractions must arise from a redistribution of cytochrome *c* lost by leakage from the mitochondria. In a recent publication Cadavid et al. [15] confirmed this view when they assessed the extent of redistribution of mitochondrial cytochrome *c* that occurs under their conditions of tissue fractionation. They found that, under the best of circumstances, 25 to 30% of the mitochondrial cytochrome *c* is redistributed among the extra-mitochondrial fractions during cell fractionation. Cadavid et al. [15] estimated that 87% and 100% of the cytochrome *c*

(W) of nuclei and microsomes, respectively, was derived from the mitochondria. These authors do not provide corrected percent distribution data in their latest report [15], but we have found that, on adjustment of their earlier data [11], there is excellent agreement between our respective results for the intracellular distribution of cytochrome *c* in liver tissue. When the cytochrome *c* (W) content found by Cadavid and Campbell [11] for each extra-mitochondrial particulate fraction is added to the value for the cytochrome *c* content of the mitochondria, the corrected values (column *c*, table 1) for the percentage distribution then fall into nearly perfect agreement with the findings of this laboratory.

We have sought to determine the basis for the differences in the percentage distribution found by our respective laboratories. Two types of experiments have been performed to compare the initial homogenizing media used by each laboratory. These experiments were constructed to determine whether: (a) either medium especially fostered the release of cytochrome *c* from mitochondria (as measured by the cytochrome *c* content of the isolated nuclei, after final sedimentation in 2.2 M sucrose); (b) either medium affected the extent of binding of free cytochrome *c* by nuclei. Emphasis was placed on the effects of the media on nuclear binding of cytochrome *c* because of the known ability of nuclei to bind that protein [16]. The results of these experiments were as follows: (a) No differences were found in the nuclei isolated by use of 0.3 M sucrose [11] or 0.25 M sucrose–3 mM $MgCl_2$–5 mM triethanolamine, pH 7.0 [3]; the yields of nuclei were 41% and 42% and the cytochrome *c* contents were 0.31 and 0.35 μg/mg protein, respectively. (b) In a second type of experiment, purified nuclei (134 mg nuclear protein) were suspended in 10 ml of each medium and equilibrated with 500 μg of cytochrome *c* (in 0.2 ml of 0.25 M sucrose) for 10 min at 25°. After sedimentation and two washings in the appropriate medium, analysis of the cytochrome *c* content of each nuclei sample showed each to have bound 275 μg of the added cytochrome *c*. Thus, there is no essential difference between the 0.3 M sucrose medium of Cadavid and Campbell [11] and that employed in our studies in regard to either an effect on the ability of nuclei to bind free cytochrome *c*, or on mitochondrial integrity during cell fractionation. We conclude that the extensive redistribution of mitochondrial cytochrome *c* found by Cadavid and Campbell [11] must stem principally from their technique of homogenization. In this connection, Cadavid and Campbell [11] used a homogenizer and pestle with a clearance of 0.10 to 0.15 mm with a speed of rotation of 2000 to 4000 rpm. The apparatus used in our work has a specified clearance of 0.19 mm and we used a speed of rotation of 1280 rpm.

The results of this work establish that the overwhelming bulk of the cytochrome *c* of liver tissue resides in the mitochondria. Though the results of our fractionations exhibit reasonable consistency, in some experiments the mitochondrial cytochrome *c* constitutes as much as 95% of the total. We have come to believe that this percentage more truly approaches the correct figure and that the average of 91% represents the occurrence of a variable minimal release of cytochrome *c* which is unavoidable by present techniques. A similar conclusion has been reached by Kadenbach [14] in work in which he has also confirmed the synthesis of cytochrome *c* by the endoplasmic reticulum. In addition, he has concluded that the magnitude of the true level of microsomal cytochrome *c* is so small that it is inestimable by conventional means of analysis but for the fact that it is contaminated by a many-fold excess of cytochrome *c* which is derived by leakage of the protein from mitochondria. By direct analysis, Kadenbach [14] found isolated microsomes to contain 4% of the total cytochrome *c* – a figure in good agreement with our results (table 1). However, by indirect mathematical analysis of the rate of labeling of cytochrome *c* of microsomes and mitochondria with [^{14}C]lysine, Kadenbach [14] estimated that the actual amount of cytochrome *c* endogenous to the endoplasmic reticulum is only 0.10% of the total. At present we cannot confirm or deny the latter estimate for the microsomal percentage of total cytochrome *c*, but our findings do establish that the mitochondria of liver tissue can be isolated and shown to contain a minimum of 90 to 95% of the total cytochrome *c*. We are presently seeking to confirm Kadenbach's estimate of the cytochrome *c* endogenous to microsomes by use of another approach.

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References

[1] N.F.G.Cadavid and P.N.Campbell, Biochem. J. 105 (1967) 443.
[2] N.McC.Davidian and R.Penniall, Fed. Proc. 25 (1968) 768.
[3] R.Penniall and N.McC.Davidian, FEBS Letters 1 (1968) 38.
[4] D.Beattie, R.Basford and S.Koritz, Biochemistry 5 (1966) 926.
[5] B.Kadenbach, Biochim. Biophys. Acta 134 (1967) 430.
[6] D.B.Roodyn, J.W.Suttie and T.S.Work, Biochem. J. 83 (1962) 29.
[7] R.Tsanev and G.G.Markov, Biochim. Biophys. Acta 42 (1960) 442.
[8] W.B.Elliott, R.D.Hayford and W.Tanski, Biochim. Biophys. Acta 88 (1964) 219.
[9] M.A.Swanson, in: Methods in Enzymology, vol. II, eds. S.P.Colowick and N.O.Kaplan (Academic Press, New York, 1955) p. 541.
[10] A.G.Gornall, C.J.Bardawill and M.M.David, J. Biol. Chem. 177 (1949) 751.
[11] N.F.G.Cadavid and P.N.Campbell, Biochem. J. 105 (1967) 427.
[12] T.E.Conover and G.Siebert, Biochim. Biophys. Acta 99 (1965) 1.
[13] W.D.Currie, N.M.Davidian, W.B.Elliott, N.F.Rodman and R.Penniall, Arch. Biochem. Biophys. 113 (1966) 156.
[14] B.Kadenbach, personal communication.
[15] N.F.G.Cadavid, M.Bravo and P.N.Campbell, Biochem. J. 107 (1968) 523.
[16] H.Beinert, J. Biol. Chem. 190 (1951) 287.