COPPER-DEFICIENT MITOCHONDRIA
SPECTROPHOTOMETRIC DETERMINATION OF CYTOCHROMES

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A critical dependence of the cytochrome oxidase content of yeast and of mammalian tissues on the copper content of the biosynthetic medium or diet was first demonstrated by Elvehjem (1931) and Cohen and Elvehjem (1934), respectively. Gallager et al. (1956) subsequently extended the latter investigation to the mitochondria isolated from the livers of rats raised on a copper-deficient diet. Analogous studies of mitochondria isolated from copper-deficient yeast have not yet been reported.

Cytochrome oxidase protein normally comprises about 8 per cent of the membrane-bound protein of rat liver mitochondria (Jacobs et al., 1966). The low level of cytochrome oxidase induced by copper deficiency could therefore be expected to result in some modification of the structure of the mitochondria membrane. Observations relevant to such an effect and to the possibility that an apo-oxidase protein is actually present in copper-deficient mitochondria (Wohlrab and Jacobs, 1967) will be described in subsequent communications. In this communication we wish to describe a spectrophotometric determination of the extent of depletion of the various cytochromes in copper-deficient mitochondria derived from both rat liver and yeast.

MATERIALS AND METHODS

Baker's yeast (s. cervisiae) were grown in a copper-free, synthetic medium (Galsy and Slonimski, 1957) under vigorous aeration using lactic acid as a carbon source. The growth medium was depleted of copper by

extraction with a layer of zinc dibenzyldithiocarbamate in CCl_4, essentially as described by Giorgio et al. (1963). This method routinely reduces the copper content of the growth medium to 0.25 - 0.50 x 10^{-6} g/ℓ. The growth medium used to obtain normal yeast cells was obtained by adding 25 x 10^{-6} g of copper ($CuSO_4$) per liter of extracted medium.

Rats were raised on the copper-deficient diet developed by Dallman and Lostkutoff (1967). About 60 days after birth the liver mitochondria were evidently deficient in cytochrome oxidase as indicated by the relative decrease of the absorption band (reduced) at 605 mμ. The mitochondria were prepared from both normal and copper-deficient livers in 0.25 M sucrose, according to the method of Lardy and Wellman (1952). The mitochondrial preparations were then depleted of endogenous cytochrome c by serial extraction with water and 0.15 M KCl and the cytochrome c recovered quantitatively from the extracts, according to the procedure described by Jacobs and Sanadi (1960a).

Optical absorption spectra were taken with the Cary Model 14M double-beam spectrophotometer, using the scattered transmission attachment (model 1462). The concentration of cytochrome c was taken as proportional to the optical absorbance at 550 mμ of the reduced cytochrome recovered from the water-salt extracts by the aforementioned procedure. The concentration of cytochrome a_3 was taken as proportional to the difference between the Δ O.D. 445-465 mμ of the succinate reduced, minus oxidized, and the succinate reduced carbon monoxide complex, minus oxidized, preparations. This difference essentially represents the contribution of cytochrome a_3 to the total absorption of cytochromes a-a_3 at 445 mμ. The contribution of the carbon monoxide complex of cytochrome a_3 to the absorption at 445 mμ is considered to be negligible (Yonetani, 1960). The concentrations of the other cytochromes were taken as proportional to the in situ optical absorbance differences between their characteristic wavelength pairs as specified by Chance and Williams (1955) and Estabrook (1958). This latter method of determining cytochrome concentra-

tions is not strictly accurate, in that variable amounts of any one cytochrome can influence the apparent shape and height of the absorption bands of the others. It may, however, be considered to give good approximate values. Protein was measured with the Biuret reagent as modified by Jacobs and Sanadi (1960b) and Minakami et al. (1962).

RESULTS

The succinate reduced, minus oxidized, difference spectra of the normal and copper-deficient rat liver mitochondria which have been depleted of endogenous cytochrome c are compared in Figures 1A and 1B. The curves clearly

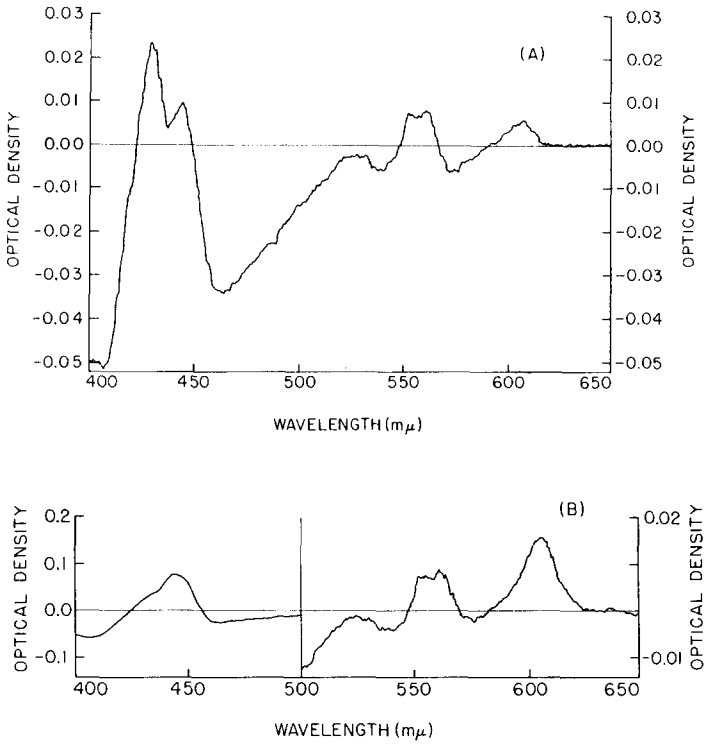

Fig. 1A. Difference spectrum of copper-deficient rat liver mitochondria depleted of endogenous cytochrome c. Sample cell contains 30 mM succinate and 4.1 mg mitochondrial protein/ml. Reference cell contains only the mitochondria.

Fig. 1B. Difference spectrum of normal rat liver mitochondria depleted of endogenous cytochrome c. Sample cell contains 30 mM succinate and 3.9 mg mitochondrial protein/ml. Reference cell contains only the mitochondria.

indicate that there is a considerable reduction of cytochromes a-a_3 relative to cytochromes b and c_1 in the copper deficient preparation. In order to determine the exact relative amounts of the various cytochromes in the two preparations, the following procedure was adopted. The preparations, depleted of endogenous cytochrome c, were adjusted to exactly equal concentrations of protein, and treated with sufficient antimycin to inhibit succinate oxidation completely. The succinate reduced, minus oxidized, difference spectra were recorded and taken as representative of that of cytochrome b alone. These data, included in Table I, show that the cytochrome b content of the mitochondria is invariant to copper-depletion. The succinate reduced, minus oxidized, difference spectra in the absence of antimycin were then recorded and used to determine the amounts of cytochromes c_1 and a-a_3 with respect to cytochrome b. Reduction with dithionite instead of succinate did not change the results. These data, included in Table 1, show that cytochrome c_1 is also invariant to copper-depletion. Finally, the succinate-reduced carbon monoxide complex, minus oxidized, difference spectra were recorded and used to determine the amounts of cytochrome a_3.

TABLE I

The Relative Distribution of Cytochromes in Normal and Copper-Deficient Rat Liver Mitochondria

Preparation[1]	Cyt b)[2]	Cyt c_1)[3]	Cyt c)[4]	Cyt a-a_3)[5]	Cyt a_3)[6]
Cu (+) I	.011	.011	0.402	0.135	
Cu (+) II	.012	.011	0.392	0.147	.061
Cu (-) I	.011	.011	0.269	0.073	
Cu (-) II	.011	.012	0.234	0.047	.021

[1] 4.1 mg protein/ml of water-salt washed mitochondria in absorption cells. [2] Δ O.D. 564-575; [3] Δ O.D. 554-540; [4] mμmoles cytochrome c per mg protein of water-salt washed mitochondria; [5] Δ O.D. 605-630; [6] Δ O.D. 445-465 (succinate reduced minus oxidized) MINUS Δ O.D. 445-465 (succinate reduced carbon monoxide minus oxidized).

The amounts of cytochrome c were determined directly from the concentrated chromatographed extracts. The data, shown in Table I, demonstrate that cytochromes c, a and a_3 have all sustained a decrease, although not equally, as a consequence of copper deficiency.

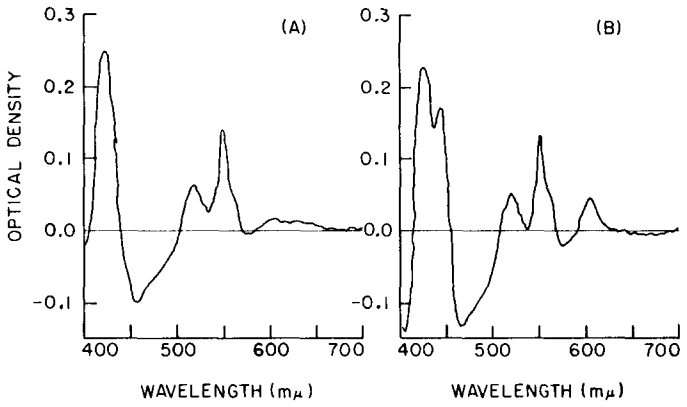

Fig. 2. Difference spectra of intact yeast cells. The sample cuvette was reduced with a trace of dithionite. The reference cuvette was maintained oxidized with a drop of 3 percent H_2O_2. A: S. Cerevisiae were grown in the copper-deficient medium, harvested at a cell count of 2.5×10^7 cells/ml, and measured in a concentration of 2.5×10^9 cells/ml. B: S. Cerevisiae were grown in the copper deficient medium supplemented with 25×10^{-6} g. copper ($CuSO_4$) per liter of medium, harvested at a cell count of 2.9×10^7 cells/ml, and measured in a concentration of 2.9×10^9 cells/ml.

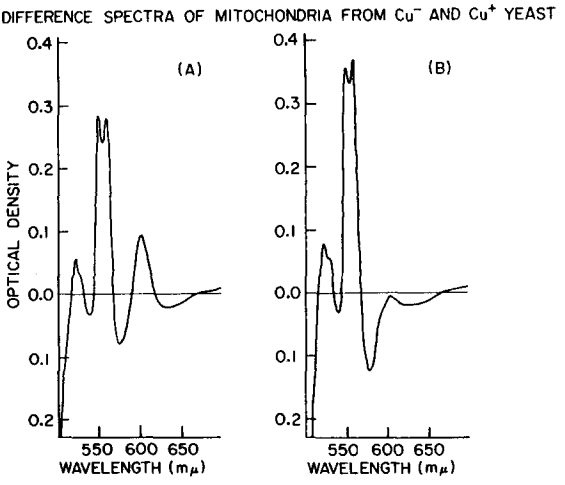

Fig. 3. Difference spectra of mitochondria derived from copper-supplemented (A) and copper-deficient (B) yeast. Sample cell reduced with a trace of dithionite.

The dithionite reduced, minus oxidized, difference spectra of normal and copper-deficient yeast cells, and of the mitochondria prepared from these cells with a French Pressure Cell are shown in Figures 2 and 3. The markedly increased absorption seen at 556 mμ in Figure 3 is presumably due to the direct reduction of cytochrome b_2 in the mitochondrial preparation by dithionite. Presumably, dithionite effects only a state of anaerobiosis in the intact cells with endogenous substrate being the reducing agent of the intracellular mitochondria. The decrease in the level of cytochrome a induced by copper-deficiency is greater than in rat liver mitochondria because of the lower level of trace copper attainable with yeast. Otherwise, the relative amounts of cytochromes b , c_1 and c in the yeast preparations were found to be analogous to those in the rat liver preparations.

DISCUSSION

The data presented in this communication indicate that the levels of cytochromes b and c_1 remain unchanged in copper-deficient mitochondria while the levels of cytochromes c and $a\text{-}a_3$ decrease, although not commensurately. The latter observation speaks against the concept of a 1:1 c:a complex suggested by King et al. (1965). However, the possibility that an apo-oxidase lipoprotein is present in copper-deficient mitochondria is suggested by the work of Wohlrab and Jacobs (1967), and if the existence of such a lipoprotein is confirmed it could account for the excess bound cytochrome c. Our observations with rat liver are somewhat in contrast to those of Gubler et al. (1957), who found the level of cytochrome c in copper-deficient swine heart to increase about 10 percent with an 80 percent decrease in cytochrome a. Additionally, we have also noticed that only a slight decrease in the level of cytochrome c in copper-deficient rat heart mitochondria accompanies a 70 percent reduction of cytochrome a. The c:a stoichiometry thus appears to be somewhat variable, but, in general, does not support the existence of an obligatory 1:1 complex. Some observations on the functional role of the cytochromes in electron transport processes in copper-deficient mitochondria are presented in the accompanying communication.

A deficiency of cytochrome oxidase in yeast has been reported under a variety of conditions other than removal of copper from the biosynthetic medium. Growth in the presence of antimycin (Ycas, 1956), in the absence of pantothenate (Okuda and Takahashi, 1967), in the absence of oxygen (Biggs and Linnane, 1963) and of respiration-deficient mutant strains (Sherman and Slonimski, 1964; Reilly and Sherman, 1965). A relative distribution of cytochromes has been reported only for yeast cells grown under low aeration, and it is similar to our findings with copper-deficient rat liver mitochondria.

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