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Mitochondria of Isolated Plant Cells (*Acer pseudoplatanus* L.)

II. COPPER DEFICIENCY EFFECTS ON CYTOCHROME *C* OXIDASE AND OXYGEN UPTAKE

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RICHARD BLIGNY AND ROLAND DOUCE
D.R.F./Biologie Végétale, Centre d'Etudes Nucléaires et Université Scientifique et Médicale de Grenoble, 85 X 38041 Grenoble Cedex, France

ABSTRACT

The effects of copper deficiency on cell culture growth, cell respiration, mitochondrial oxidative properties, and electron transport chain have been studied with suspension-cultured sycamore cells (*Acer pseudoplatanus* L.). Within the range of the copper concentration studied (0.1-25 μg/l of culture medium), the mean rate of cell division is independent of copper concentration. An initial copper concentration lower than 2 μg/l limited the maximum density of population reached at the stationary phase of growth.

On a protein basis, the uncoupled O_2 uptake rates were about the same for normal and copper-deficient cells. In contrast, the half-maximal inhibition of O_2 uptake rate was obtained at greater KCN concentration in the normal cells (20 μM) compared to copper-deficient cells (2 μM). Similar results were obtained with the normal and copper-deficient sycamore cell mitochondria.

In the copper-deficient mitochondria, the concentration of the cytochrome aa_3 was less than 0.02 nmol/mg mitochondrial protein or $^1/_{20}$ of the normal rate. The *b*- and *c*-type cytochrome content was invariant with copper depletion. It appeared that cytochrome aa_3 is present in large excess in normal cells. This work also indicated that cytochrome *c* is a very mobile molecule.

The Cyt *c* oxidase complex, or Cyt aa_3 (EC 1.9.3.1) is a structural element of the mitohondrial inner membrane, the terminal enzyme of the electron transport chain, and an integral part of coupling site III. It has been isolated with detergents as a multipeptide aggregate (six subunits) containing two hemes and two atoms of copper (14, 22). The two hemes in the Cyt aa_3 are functionally distinct. The first one, heme *a*, reacts with Cyt *c* (10); the other one, heme a_3, reacts with O_2 and ligands CO (18). Furthermore, Cyt aa_3 spans the mitochondrial membrane asymmetrically (9), heme *a* is on the side facing the outer membrane whereas heme a_3 is situated on the matrix side, and it has been demonstrated that there is a topological sequence *a*-cu-cu-a_3 (21).

Previous studies using the spectrophotometric method have shown that copper deficiency in yeast cells (13, 15) inhibits Cyt aa_3 appearance. Analogous studies of mitochondria isolated from copper-deficient plant cells have not yet been reported. In this communication we describe the evolution of the various Cyt in copper-deficient mitochondria prepared from suspension-cultured *Acer pseudoplatanus* L. cells. We also compare the physiological properties of these copper-deficient mitochondria with those obtained from normal cells.

MATERIALS AND METHODS

Cells and Culture Conditions. The strain of *A. pseudoplatanus* L. was a gift of J. Guern. The basic nutrient medium was prepared according to Lamport (16) modified by Lescure (19). Reagent grade chemicals were used. Copper-free water was obtained by passing twice distilled H_2O through a column of Chelex 100 (acidic form) at a flow rate of 2 ml/cm^2·min. This method reduced the copper content of the culture medium to less than 0.1 μg/l. The growth medium used to obtain copper-deficient cells contained 0.5 μg of copper/l of medium. The growth medium used to obtain normal sycamore cells contained 25 μg of copper (100 μg $CuSO_4 \cdot 5H_2O$)/l of medium.

Cell suspensions were cultured in a phytostat for automatic mass culture of plant cells in liquid medium. This apparatus described in a previous publication (2) allowed the culture of 20 l/cell suspension. The automatic recording of cell suspension growth was carried out by means of turbidity measurement (2), since it was shown that cell number and culture turbidity were closely correlated throughout the exponential phase of growth.

Preparation of Mitochondria. Unless otherwise stated, sycamore cells in very good physiological state were harvested just at the end of the exponential phase of growth. Standard techniques were used for the preparation of the mitochondria (3). Typical preparations started with 1 kg of packed sycamore cells which were disrupted for 15 sec with a Moulinex mixer 66 (Alençon, France). The grinding medium used consisted of 0.3 M mannitol, 1 mM EDTA, 20 mM MOPS buffer (pH 7.4), 0.1% defatted BSA, and 0.05% cysteine. Mitochondria were purified on sucrose gradient (6). The yield of mitochondria was around 30 mg mitochondrial protein/preparation.

Oxygen Uptake. O_2 uptake was measured at 25 C in a 3-ml sealed vessel using a Clark O_2 electrode (Beckman, O_2 analyzer, fieldlab) as described by Estabrook (8). The reaction medium for the cells is their culture medium. The reaction medium (medium A) for the mitochondria contained: 0.3 M mannitol, 10 mM KCl, 5 mM $MgCl_2$, and 10 mM K-phosphate buffer. The pH was adjusted to 7.2. The O_2 concentration in the air-saturated medium at 25 C was taken as 240 μM (8).

Split Beam Spectrophotometry. This was performed with the Aminco DW-2 spectrophotometer. The concentrations of the different Cyt were measured at room or liquid N_2 temperature (77 K) from reduced minus oxidized difference spectra. Mitochondrial preparations were reduced by succinate. The wavelengths selected for measurements and the extinction coefficients were those given by Chance and Williams (5) and Lance and Bonner (17). The magnitude of the low temperature enhancement was measured according to Lance and Bonner (17).

General Methods. The copper contents were measured by atomic absorption without flame (carbonrod) after formation of a complex by ammonium pyrrolidine dithiocarbamate (APDC) and extraction by methylisobuthylketone (MIBK). Mitochondrial protein concentration was determined by the method of Lowry *et al.* (20) with BSA as standard. Total cell protein was measured according to the method of Nessler after elimination of the soluble N by precipitation and washing of the proteins with 10% trichloroacetic acid.

RESULTS AND DISCUSSION

Dependence of Rate of Cell Division on Copper Concentration. Figure 1 indicates that, within the range of copper concentrations studied, the mean rate of cell division is independent of copper concentration. However, an initial concentration of copper lower than 2 μg/l limits the maximum density of sycamore cells. These results strongly suggest that copper at a concentration lower than 2 μg/l is the limiting factor of the total growth of sycamore cells. In order to confirm the influence of copper as a limiting factor of cell population growth, the following experiment was carried out. Sycamore cells were inoculated in a medium at a low initial concentration of copper (0.5 μg/l). The growth of cells was followed until stationary phase of growth was reached (Fig. 1). Addition of copper 5 days after growth had ceased caused a large increase in the cell number to the level of the normal stationary phase. Before the growth started, a lag phase of about 2 days was noticed. Consequently, this experiment indicates that the arrest of cell division at stationary phase is due to copper deprivation.

Quantitative Determination of Cytochromes. Figure 2 shows the room temperature difference spectra obtained with mitochondria from normal and copper-deficient sycamore cells. The two spectra show a clear identification of the Cyt after reduction of the mitochondrial preparation by succinate. In the case of normal mitochondria, the α band of Cyt aa_3 is located at 603 nm, the α band of Cyt *c* at 550 nm, and the α band of Cyt *b* appears as a shoulder centered around 560 nm. The Soret region is dominated by the classical absorption peaks of Cyt *b* at 428 nm and Cyt aa_3 at 445 nm. These observations are in good agreement with Lance and Bonner (17). In the case of copper-deficient mitochondria we observe only a striking reduction of the Cyt aa_3 peaks (603 and 445 nm). For example, in the Soret region Cyt aa_3 appears as a small shoulder on the longer wavelength side of the Cyt *b* peak at 428 nm.

The difference spectra of normal mitochondria shown in Figure 3 are typical of plant mitochondria when examined at liquid N_2 temperature. Cyt aa_3 has a peak at 599 nm. The α band of Cyt *c* appears at 549 nm. The shoulder at 560 nm representing the *b* Cyt complex in Figure 2 has been split into three peaks absorbing at 562, 557, and 553 nm. In the Soret region, the Cyt aa_3 shows a typical double peak at 444 and 437 and the *b* + *c*-type Cyt give a common peak at 423 nm. Again in the case of copper-deficient mitochondria, we observe only a striking reduction in the Cyt aa_3 peaks. The α band of the Cyt *c* is not affected. Figure 4 shows that the Cyt *b* is also not affected by the copper deficiency.

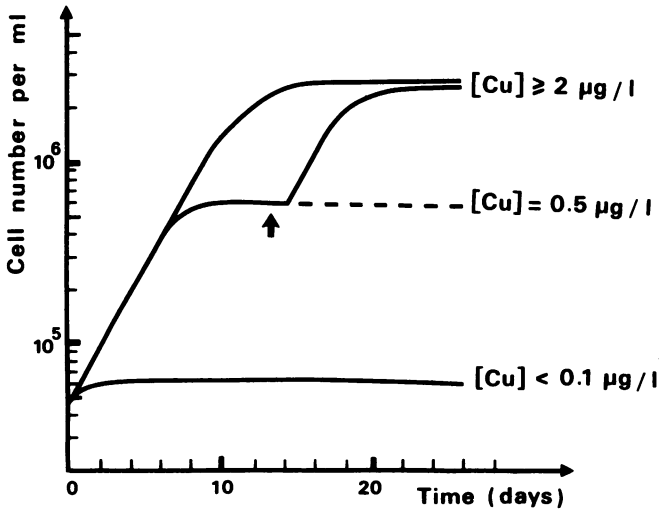

FIG. 1. Influence of initial copper concentration on growth of suspension-cultured sycamore cells. Inoculum (800 ml, 2 × 10^6 cells/ml) was taken from copper-deficient cell suspensions in exponential phase of growth. Final volume of the culture: 19 liters. Arrow shows an injection of copper (25 μg/l of culture).

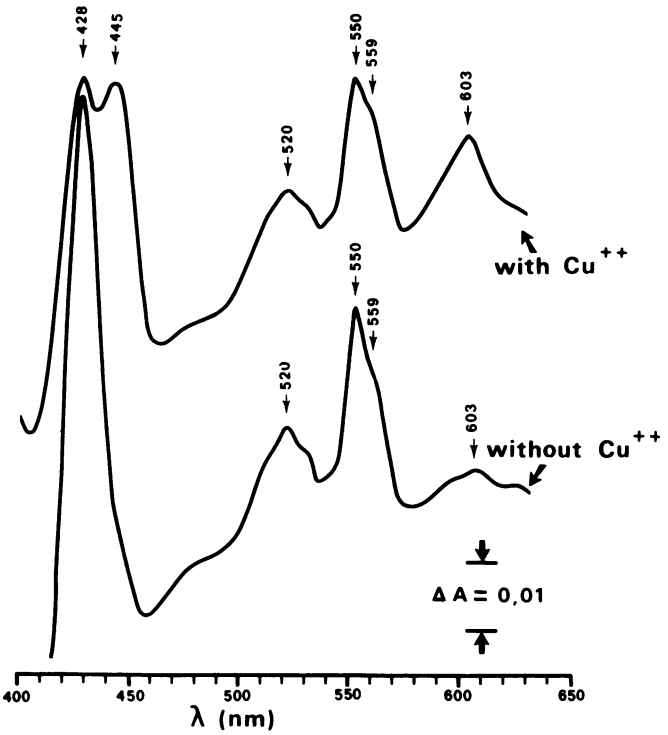

FIG. 2. Difference spectra of normal and copper-deficient sycamore cell mitochondria at room temperature (25 C). Succinate (20 mM) reduced minus aerobic. Mitochondria were suspended in medium A (see under "Materials and Methods"). Optical path: 10 mm (quartz cuvettes). Mitochondrial protein: with copper, 2.2 mg/ml; without copper, 2.8 mg/ml.

The concentrations of the various Cyt for normal and copper-deficient mitochondria are given in Table I. Expressed in terms of nmol/mg of mitochondrial protein, the concentration of the Cyt aa_3 is dramatically lower in the copper-deficient mitochondria: less than 1/20 of the normal rate. In contrast, the *b*- and *c*-type Cyt content of the sycamore mitochondria is invariant to copper depletion.

Chemical removal of copper from purified Cyt aa_3 does not alter its spectrum (23) suggesting, in our experiments, the absence of the apoenzyme and/or heme *a* + a_3 in copper-deficient sycamore cell mitochondria since the spectrum was changed. However, very recently Keyhani and Keyhani have shown that in *Candida utilis* yeast cells, the six subunits of Cyt aa_3 were found to be present in copper-deficient mitochondria (14, 15). In these conditions it is very likely that the striking reduction in the Cyt aa_3 peaks in copper-deficient plant mitochondria is attributable to the absence of heme *a* + a_3.

When the copper-deficient cells are inoculated in a nongrowing medium (without nitrate) in the presence of copper (25 μg/l), there is no formation of the Cyt aa_3. When the copper-deficient cells are inoculated in a growing medium (with nitrate) in the presence of copper (25 μg/l), the increase of Cyt aa_3 peaks is very slow. It is only after one generation time (when the cell number has doubled) that Cyt aa_3 is readily detectable; at this stage the amount of Cyt aa_3 is the half of the amount found in normal mitochondria. In these conditions, the appearance of spectrophotometrically detectable Cyt aa_3 in copper-deficient sycamore cells is a slow process which is governed by the synthesis of new mitochondrial material. Since the turnover of sycamore cell mitochondria is very slow (Bligny and Douce, unpublished data) full recovery of normal Cyt aa_3 amount would be expected to be slow. These data seem to indicate that

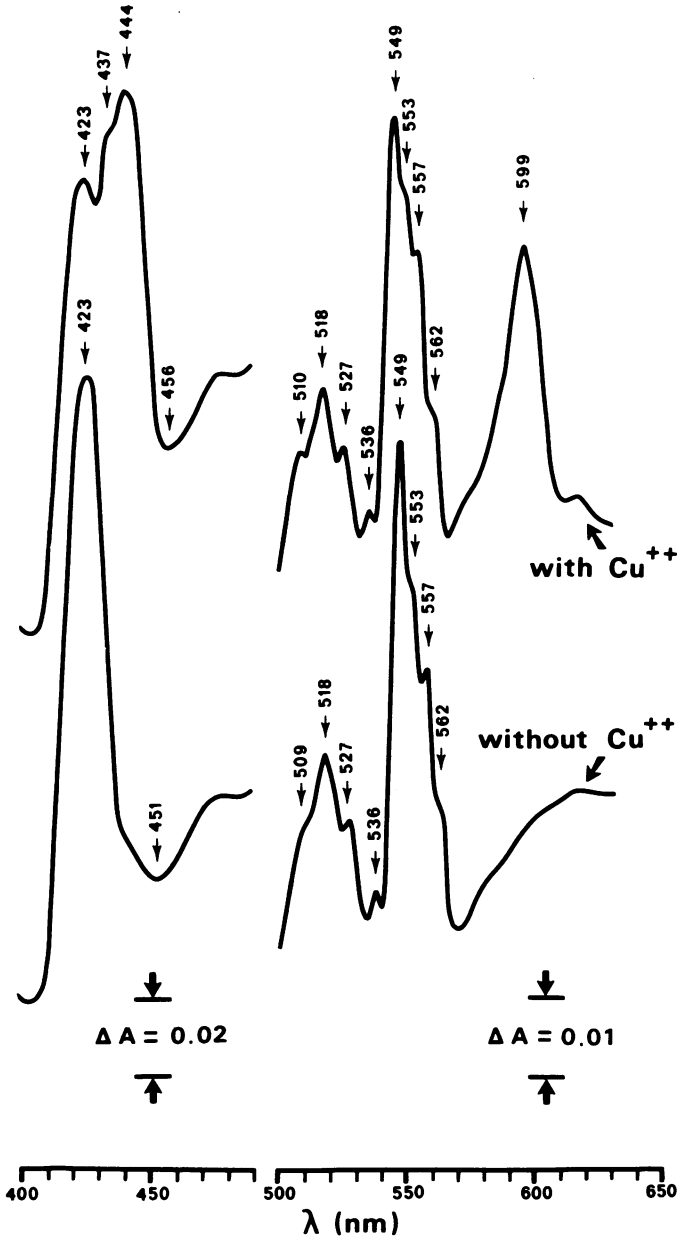

FIG. 3. Difference spectra of normal and copper-deficient sycamore cell mitochondria at liquid N_2 temperature (77 K). Succinate (20 mM) reduced minus aerobic. Mitochondria were suspended in medium A (see under "Materials and Methods"). Optical path: 2 mm (Plexiglas cuvettes). Mitochondrial protein: with copper, 4.2 mg/ml; without copper, 4.8 mg/ml.

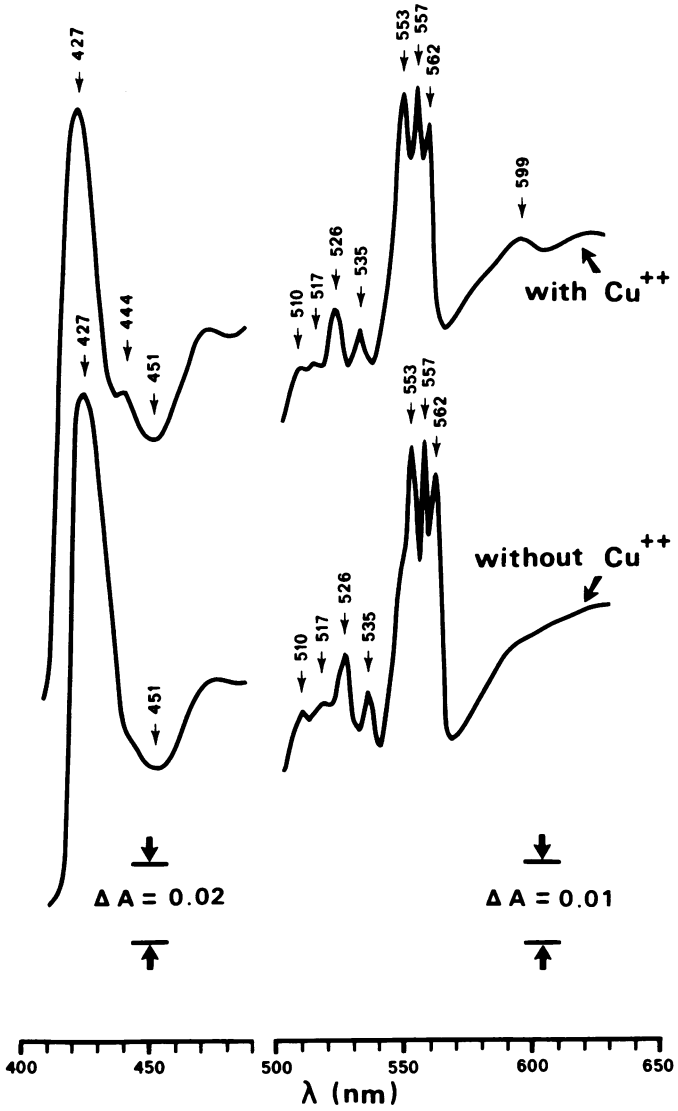

FIG. 4. Difference spectra of normal and copper-deficient sycamore cell mitochondria at liquid N_2 temperature (77 K). Succinate (20 mM) aerobic with antimycin A (5 μg/ml) minus aerobic without antimycin A. Mitochondria were suspended in medium A (see under "Materials and Methods"). Optical path: 2 mm (Plexiglas cuvettes). Mitochondrial protein: with copper, 5.4 mg/ml; without copper, 5.9 mg/ml.

new mitochondria synthesis is necessary for the reconstitution of Cyt aa_3 and that the mitochondria from copper-deficient cells are not modified after addition of copper.

Respiration Rates. Typical respiration rates for normal and copper-deficient sycamore cells are shown in Figure 5. Both types of cells were harvested just at the end of the exponential phase of growth. Although the Cyt aa_3 content is considerably reduced in copper-deficient cells, the O_2 uptake rates are about the same for normal and copper-deficient cells even after adding an uncoupler (FCCP[1]) in the medium. However, we have observed (Fig. 6) that half-maximal inhibition of the O_2 uptake rate is obtained at greater KCN concentration in the normal cells (20 μM) compared to copper-deficient cells (2 μM). It has to be stressed that the final and strong inhibition obtained with cyanide is about the same for both types of cells. Our result indicates, in contrast with yeast cells (13), that in the absence of copper, sycamore cells do not develop a branched electron chain on the substrate side of Cyt *b* in order to consume

Table I. Cytochrome components of the respiratory chain in normal and copper-deficient sycamore cells mitochondria as determined at liquid nitrogen temperature (77 K).

The concentrations are measured from succinate reduced minus aerobic and from succinate plus antimycin A aerobic minus-aerobic difference spectra. All table values are mean values of 5 experiments for each type of mitochondria. Absorption coefficient of CHANCE and WILLIAMS (5) and intensification factor of LANCE and BONNER (17) were used. Cyt aa_3 : cytochrome *c* oxidase.

type of mitochondria	Concentration in electron carriers: cyt aa_3	cyt *b* 553	cyt *b* 557	cyt *b* 562	cyt *c*
	nmoles per mg protein				
with copper	0.40	0.28	0.28	0.25	0.55
without copper	<0.02	0.26	0.27	0.24	0.53

[1] Abbreviation: FCCP: carbonyl cyanide *p*-trifluoro methoxy phenylhydrazone.

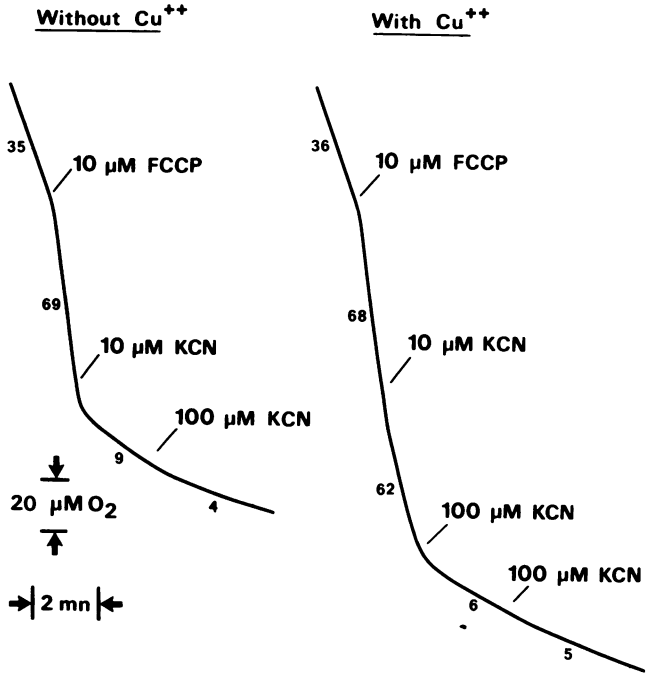

FIG. 5. Effect of FCCP and KCN on O_2 uptake by normal and copper-deficient sycamore cells. Numbers on traces refer to nmol O_2 consumed/min·mg cellular protein.

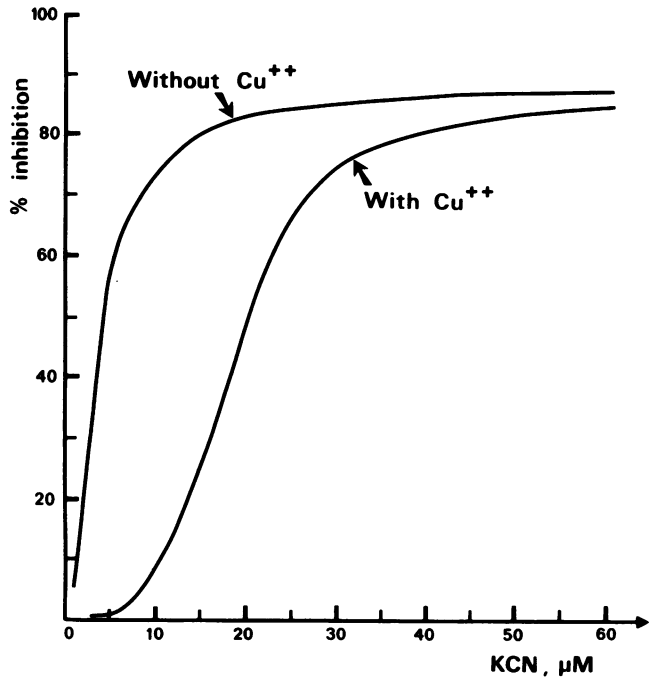

FIG. 6. Effect of cyanide concentration on normal and copper-deficient sycamore cells respiration. *y* axis: per cent inhibition of the uncoupled cell respiration (see Fig. 5).

molecular O_2 (1).

The quality of the mitochondrial preparations can be appraised rapidly by the measurement of certain characteristic parameters. Respiratory activity is shown in Figure 7 which illustrates several O_2 electrode traces obtained from sycamore cell preparations (normal and copper-deficient cells). These traces show that on a protein basis the rates of O_2 uptake in state 3 are about the same for normal and copper-deficient mitochondria. The comparison of the ADP/O ratio and respiratory control of the two types of mitochondria leads to the same

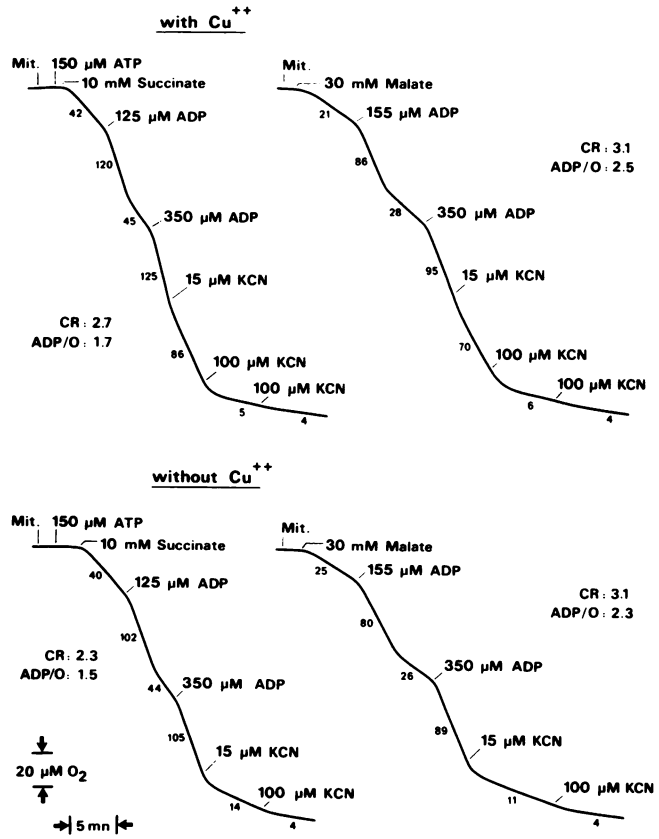

FIG. 7. Oxidation of succinate and malate by normal and copper-deficient sycamore cell mitochondria. Concentrations given are final concentrations in reaction medium. Numbers on traces refer to nmol O_2 consumed/min·mg mitochondrial protein. CR: respiratory control index.

conclusion. In contrast, as found for the respiration of intact cells, the half-maximal inhibition of O_2 uptake rate is obtained at greater cyanide concentration in the normal mitochondria compared to the copper-deficient mitochondria.

From the measurements of the rates of succinate oxidation (Fig. 7) and from the values of Table I it is also possible to determine the turnover number of Cyt aa_3 in isolated mitochondria. The turnover number of Cyt aa_3 in copper-deficient mitochondria (above 400 sec^{-1}) is more than 20 times higher than in non-copper-deficient mitochondria (about 20 sec^{-1}). It is also interesting to note that the value found for copper-deficient mitochondria is of the same order of magnitude as the highest value found with purified Cyt aa_3 (24). However, the turnover number of Cyt *c* is invariant to copper depletion.

In contrast to what was expected, these observations indicate clearly that the low amount of Cyt aa_3 present in copper-deficient mitochondria does not contribute to limit the electron flow at the level of the inner mitochondrial membrane. It appears also, in good agreement with early suggestions (4, 7), that the Cyt aa_3 is in very large excess in plant mitochondria. It is apparent that existing models of electron transport based on a stoichiometry of 1 molecule of Cyt *c*/molecule of Cyt aa_3 (24) may have to be revised to account for these experimental findings. Jung and Devault (12) have shown that the Cyt aa_3 either is totally immobilized in the membrane or that it shows only limited rotational diffusion around a single axis coinciding with the symmetry axis of heme a_3. This implies that the Cyt *c* which carries the electron flow between Cyt c_1 and Cyt *a* (18) must be extremely mobile on the surface of the outer face of the inner membrane.

Finally these observations also indicate that it is not the rate

of respiration which limits the cell population growth in copper-deficient medium. It has to be pointed out that copper is also involved in other enzymes such as superoxide dismutase (11) and polyphenoloxidase. For example, we have found (unpublished data) that superoxide dismutase activity is considerably reduced in copper-deficient cell mitochondria.

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