Effect of Iron Deficiency on the Respiration of Sycamore (Acer pseudoplatanus L.) Cells

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The effects of iron deficiency on cell culture growth, cell respiration, mitochondrial oxidative properties, and the electron transport chain were studied with suspension-cultured sycamore (Acer pseudoplatanus L.) cells. Iron deprivation considerably decreased the initial growth rates and limited the maximum density of the cells. Under these conditions, the cells remained swollen throughout their growth. The absence of iron led to a steady decline in the uncoupled rate of O2 consumption. When the uncoupled rate of O2 uptake closely approximated the respiratory rate, the cells began to collapse. At this stage, the level of all the cytochromes and electron paramagnetic resonance-detectable Fe-S clusters of the mitochondrial inner membrane were dramatically decreased. Nevertheless, it appeared from substrate oxidation measurements that this overall depletion in iron-containing components solely disturbed the functioning of complex II, whereas neither complexes I, III, or IV, nor the machinery involved in ATP synthesis, was apparently impaired in iron-deficient mitochondria. However, our results suggest that the impairment of complex II resulted in a strong reduction of the overall capacity of the mitochondrial electron transport chain, which was responsible for determining the rate of endogenous respiration in sycamore cells. Finally, this situation led to a depletion of various energy metabolites that could contribute to the premature cell death.

The great need for iron in biological systems is a consequence of its irreplaceable role as a catalyst for many intracellular reactions. An important example is mitochondrial electron transfer via Cyt s and iron-sulfur (Fe-S) proteins. Thus, Ohnishi et al. (1971) demonstrated that low iron content in the growth medium of yeast cultures significantly decreased the mitochondrial content of many EPR-detectable Fe-S clusters. In addition, severe iron deficiency results in a large decrease in the activity of mitochondrial respiratory chain dehydrogenases (succinate- and NADH-ubiquinone oxidoreductases) in skeletal muscle mitochondria (Maguire et al., 1982; Ackrell et al., 1984), leading to a decrease in mitochondrial respiratory activity. Likewise, Carty et al. (1986) provided evidence for the perturbation of mitochondrial composition in muscle by iron deficiency: Cyt c concentration and Cyt oxidase activity were decreased by 50% and succinate dehydrogenase and NADH dehydrogenase activities were decreased by 78% in iron-deficient muscle. However, there is little experimental information regarding perturbation of respiration in plants by iron deficiency. The concepts of iron function in cell respiration are being explored both in animal systems and in yeast, providing an important reference point. Nevertheless, crucial differences in the impact of iron deficiency on cell respiration between plants and animals may exist and thus we must not hastily embrace the findings using mammalian cells until key experiments on plant cells have been completed.

In this context, we undertook the present study to analyze the effects of iron deficiency on plant cell culture growth and respiratory metabolism. Plant cell-suspension cultures rapidly generate large amounts of cell material that offer a useful tool for studying plant cell physiology.

MATERIALS AND METHODS

Biological Material

Sycamore (Acer pseudoplatanus L.) cells were grown at 20°C as a suspension in a nutrient medium (Lamport's media containing 10 μM FeSO4 supplied with an equimolar amount of Na2-EDTA) according to Bligny and Leguay (1987). The cell suspensions were maintained in exponential growth by weekly subculture. The culture medium was kept at a volume of 0.3 L and stirred continuously at 60 rpm. Cells were harvested from the culture medium, rinsed three times by successive resuspension in fresh culture medium devoid of iron, and placed at zero time into flasks containing iron-free culture medium. Chemicals used for media preparation were chosen from readily available sources for having low amounts of iron contamination. In addition, all culture flasks were soaked overnight in 5 M HCl to remove contaminating iron.

At each time point, cells were harvested for the measurement of various cell parameters including cell wet weight (culture sample because of the formation of numerous suction, 20 s; pressure of suction, 2 × 104 Pa) and washed twice with distilled water to ascertain cell wet weight) and cell dry weight (after drying at 100°C for 1 h).

Cell Number

The direct counting of cells under the microscope appeared to be inaccurate for determining the cell number in any cell-suspension sample because of the formation of numerous spheroplasts and cell debris.

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Abbreviations: EPR, electron paramagnetic resonance; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone.
clumps of cells during the growth. Since classical procedures, including maceration in 8 to 10% aqueous chromic trioxide solutions as described by Bligny and Leguay (1987), were unsuccessful in improving cell dispersion, we decided to prepare protoplasts according to the method described below. Digestion of the cell walls resulted in well-separated protoplasts that were easily counted under the microscope using a Nageotte cell.

However, the number of protoplasts could not be directly related to the number of cells present in the initial sample because the final yield of protoplasts might be less than 100%. To overcome the problem, we undertook series of experiments on both cells and protoplasts: amounts of proteins and various enzymic activities such as aconitase, 6-phosphogluconate dehydrogenase, and fumarase activities were measured as described below. Each measure was performed on three samples of cells harvested from the same culture medium and on the corresponding protoplasts. Results were averaged and expressed per g of wet weight in the case of cells (Rc) or per million protoplasts (Rp). The ratio Rc/Rp corresponds to the number of cells (in millions) per g of wet weight. It was calculated for the four parameters we used (proteins, enzymic activities) and almost identical values were obtained (with so usually less than 10%). This allowed us to express all of our data on a cell unit basis.

**Preparation of Protoplasts**

Cells (20 g) were suspended in 200 mL of the digestion medium containing the culture medium with 0.45 M mannitol (final osmolarity 0.5), 1% (w/v) cellulase (Onozuka RS; Yakult Pharmaceutical Co., Nishinomiya, Japan), and 0.1% (w/v) pectolyase Y-23 (Seishin Pharmaceutical Co., Nishinomiya, Japan) adjusted to pH 5.7. Cells were incubated with constant shaking using a reciprocal shaker (20 strokes/min) at 25°C. After 1 h, protoplasts were collected by centrifuging at 150g for 10 min and then washed twice with 50 mL of medium A containing 0.45 M mannitol, 10 mM KCl, 5 mM MgCl₂, 5 mM phosphate buffer, pH 7.2, 0.1% (w/v) defatted BSA. Mitochondrial O₂ uptake was measured in the presence of various respiratory substrates and appropriate cofactors: 2 mM NADH + 300 μM CaCl₂, 10 mM citrate or 10 mM isocitrate + 500 μM malate + 300 μM ATP + 500 μM NAD⁺, 10 mM succinate + 300 μM ATP; 10 mM pyruvate + 10 mM α-oxoglutarate + 20 mM malate in the presence of 300 μM ATP, 300 μM thiamine pyrophosphate, and 500 μM NAD⁺. KCN-sensitive Cyt c-dependent O₂ uptake was measured in osmotically shocked mitochondria in the presence of 30 μM Cyt c and 8 mM ascorbate (Douce et al., 1987).

**Preparation of Extracts**

Cells (1 g wet weight) or protoplasts (30 × 10⁶ units) were suspended in 10 mL of medium B containing 50 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 2 mM DTT, 1 mM EDTA, 0.025% (v/v) Triton X-100, 1% (w/v) insoluble PVP (M₆₉ about 25,000; Serva), 0.5 mM PMSF and were disrupted by sonication for 30 s (20 kHz, 80 W; Sonimass 250 T). After stirring in a Potter-Elvehjem homogenizer, samples were centrifuged at 48,000g for 30 min. The supernatant comprised the crude extract. All procedures were carried out at 4°C under argon. For perchloric acid extraction, cells (9 g wet weight) were quickly frozen in liquid nitrogen to avoid ATP destruction and ground with 1 mL of 70% (w/v) perchloric acid. The frozen powder was then placed at −10°C and subsequently thawed. The thick suspension thus obtained was centrifuged at 10,000g for 10 min to remove particulate matter. The supernatant was neutralized with 2 M KHCO₃ to about pH 6.0 and centrifuged at 10,000g to remove KClO₃. Next, trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid was added at the final concentration of 60 mM to chelate free divalent metal ions. The resulting supernatant was lyophilized and stored in liquid nitrogen. For the 31P NMR measurements, this freeze-dried material was redissolved in 2 mL of 40 mM Hepes buffer (pH 7.8) containing 10% D₂O.

Mitochondria were resuspended in medium B at the final concentration of 5 mg protein/mL. After vortex mixing, the mitochondria were left on ice for 10 min to ensure complete lysis and then centrifuged at 48,000g for 15 min at 4°C. The supernatant comprised the mitochondrial extract.
**Measurement of Enzymic Maximum Activities**

Enzymic activities were measured on cell, protoplast, or mitochondria crude extracts. Aconitase (EC 4.2.1.3) activity was assayed by the coupled assay of Rose and O'Connell (1967), in which NADP+ reduction is measured, or by monitoring the formation of cis-aconitate at 240 nm as a function of time (Racker, 1950). Fumarase (EC 4.2.1.2) activity was assayed by recording the formation of fumarate at 240 nm (Hill and Bradshaw, 1969). 6-Phosphogluconate dehydrogenase activity was assayed according to Simcox et al. (1977).

**Protein Measurements**

Cell, protoplast, and mitochondria protein contents were determined according to the method of Lowry et al. (1951) using BSA as standard. Prior to analysis, cell samples (100 mg wet weight) and protoplast aliquots (approximately 10 mg protein) were suspended in the presence of deoxycholate (100:5:5, w/v) in Na2CO3 and NaOH. After 6 h at 4°C, samples were centrifuged (200g, 2 min) and proteins were determined in the supernatant. Total mitochondrial proteins were measured in the presence of deoxycholate 0.04% (w/v).

**Polar Lipid Measurements**

Total cell lipids were extracted according to the method of Bligh and Dyer (1959) after preparation of protoplasts and fixation in boiling water for 10 min. Suitable lipid aliquots were chromatographed in two dimensions on Silica Gel precoated TLC plates (Merck). A solvent system of chloroform:methanol:water (65:25:4, v/v) was used in the first development and chloroform:acetone:methanol:acetic acid:water (100:40:20:20:10, v/v) was used in the second development. Polar lipids were located under UV light after spraying the plates with anilinonaphthalene sulfonate (0.2% [w/v] in methanol). Fatty acid methyl esters were extracted with hexane and analyzed by GC (CP-Sil 88 column, Delsi 121 flame ionization detector, Delsi Enica integrator) at 210°C. Quantitative analyses of fatty acids and their parent lipids were performed according to the method of Allen and Good (1971).

**Measurement of the Suc and Starch Pool**

Cell samples (100 mg wet weight) were dropped into 3 mL of ice-cold 0.5 M NaOH and disrupted by sonication (20 s, 40 MHz, 80 W; Sonimass 250 T). Once vigorously stirring in a Potter-Elvehjem homogenizer, samples were neutralized with 10 M HCl and fractions of 1 mL were removed for starch determination. Portions of 500 μL were then incubated for 1 h at 35°C with 500 μL of amyloglucosidase (EC 3.2.1.3) (Sigma) (1 mg/mL in sodium acetate, pH 4.6). Glc from starch hydrolysis was determined according to the method of Bergmeyer et al. (1974).

For Suc determination, NaOH extracts were centrifuged for 10 min at 20,000g and 200 μL of the supernatant was incubated for 1 h at 25°C with 200 μL of invertase (EC 3.2.1.26) (Sigma) (5 mg/mL in sodium acetate, pH 4.6). Glc and Fru from Suc hydrolysis were determined according to Bergmeyer et al. (1974) and Bernt and Bergmeyer (1974).

**Iron Status Determination**

Total cellular iron contents were determined with a Perkin-Elmer model 2380 atomic absorption spectrophotometer. Prior to analysis, cell-suspension aliquots (5 g wet weight) were lyophilized and suspended in 20 mL of pure HNO3.

Mitochondrial iron contents were measured on wet ashed samples (Beinert, 1978) to which 0.75 mL of saturated sodium acetate solution, 1.7 mL of distilled water, 0.1 mL of sodium ascorbate (5% in water), and 0.4 mL of bathophenanthroline disulfonate (0.1% in water) were successively added (Meyer et al., 1986). After 1 h, the A335 was measured and the concentration of iron was calculated using a molar extinction coefficient of 22,140 M⁻¹ cm⁻¹ for the iron-bathophenanthroline complex (Blair and Diehl, 1961).

**EPR Measurements**

EPR measurements were recorded on a Varian E 109 spectrometer coupled to a Hewlett-Packard 9826 calculator. The samples were cooled with a liquid-helium transfer system (Oxford Instrument, ESR 900) for temperatures down to 4.2 K. Temperature was monitored with a gold-iron/chromel thermocouple about 2 cm below the bottom of the EPR sample in the flowing helium gas stream. The magnetic field was calibrated using a Varian gaussmeter. Samples of purified mitochondria (180 μL, 4–16 mg of protein), either reduced by succinate, pyruvate, and 15 mM dithionite, or oxidized by O2 or 15 μM peroxodisulfate, were placed in EPR quartz tubes, frozen rapidly in liquid N2, and stored at 77 K until assayed.

**Dual-Wavelength Spectrophotometry**

Mitochondrial Cyt analyses were performed with an Amino DW-2 spectrophotometer. The concentrations of the different Cyt were measured at room or liquid N2 temperature using dithionite-reduced minus oxidized difference spectra. The wavelengths selected for measurements and the extinction coefficients were those given by Chance and Williams (1955) and Lance and Bonner (1968). The magnitude of the low-temperature enhancement was measured according to Lance and Bonner (1968).

**31P NMR**

A Bruker NMR spectrometer (AMX 400) equipped with a 10-mm multinuclear probe tuned at 162 MHz was used to characterize the various intracellular pools of phosphorous compounds known to be present in plant cells (Gadian et al., 1979; Roberts et al., 1980). 31P NMR spectra were recorded on neutralized cell perchloric acid extracts as described by Bigny et al. (1990).


RESULTS AND DISCUSSION

Effect of Iron Deficiency on Sycamore Cell Growth

Figure 1 shows the increase in cell number (Fig. 1a) and wet weight (Fig. 1b) during the growth of sycamore cells in liquid medium either containing iron or devoid of iron. It is obvious that throughout the growth in both types of cultures, cell number and wet weight were not correlated. A marked difference was apparent during the lag and stationary phases of growth. During the lag phase, the cell number remained constant whereas wet weight increased significantly. Likewise, and as previously shown (Bilgny, 1977), during the stationary phase of growth the cell number also remained constant whereas wet weight continued to increase slowly.

In the case of control cells, the cell number doubling time was 40 to 48 h after a lag phase of approximately 2 d and the maximum density of sycamore cells was attained after 7 to 8 d of growth (Fig. 1a). Cell wet weight increased from 21 to 37 mg/10^6 cells during the lag phase, decreased from 37 to 21 mg/10^6 cells during the exponential phase of growth, and increased again from 21 to 55 mg/10^6 cells during the stationary phase (Fig. 2a). In addition, both proteins and polar lipids (reflecting cytoplasmic volume and cell membrane expansion) increased during the nondividing phases and then decreased when the cell divisions occurred (Table I). These results indicate that cell volume increased during the lag and stationary phases and decreased during the exponential phase of growth. In this context, light micrographs obtained from suspension-cultured sycamore cells showed that the mean cell diameter was about 40 μm during the lag and stationary phase of growth, whereas the dividing cells exhibited a roughly circular shape and a rather small size (25-30 μm diameter) together with a relatively small vacuolar system (not shown). On the other hand, the variations of the cell dry matter (Fig. 2b), which exhibited a time course of evolution identical to that of the cell wet weight, may be partially related to the marked fluctuations of the size (25-30 μm diameter) together with a relatively small vacuolar system (not shown). On the other hand, the variations of the cell dry matter (Fig. 2b), which exhibited a time course of evolution identical to that of the cell wet weight, may be partially related to the marked fluctuations of the

FIGURE 1. Effects of iron deficiency on growth of suspension-cultured sycamore cells. Inocula (50 mL, 2.2 × 10^6 cells/mL) were harvested from normal cell suspensions at the end of the exponential phase of growth. The final volume of the culture was 300 mL. Cells were grown at 20°C in a nutrient medium supplied with 10 μM Fe-EDTA (+Fe, standard conditions) or devoid of iron (−Fe). Cell-suspension samples were regularly taken from both types of cultures to determine the number of cells (a) after measurement of the wet weight (b) as described in “Materials and Methods.” Both characteristics were expressed per mL of culture media. Data are from one representative experiment that has been reproduced six times with similar results. L, D, and S, Lag, dividing, and stationary phases of cell growth, respectively.

Table I. Evolution of various biochemical characteristics during cell growth in the presence or absence of iron

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Days in Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (μg/10^6 cells)</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>475</td>
</tr>
<tr>
<td>Iron deficient</td>
<td>475</td>
</tr>
<tr>
<td>Glycerolipids (μg/10^6 cells)</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>60</td>
</tr>
<tr>
<td>Iron deficient</td>
<td>60</td>
</tr>
<tr>
<td>Starch + Suc (μg/10^6 cells)</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>325</td>
</tr>
<tr>
<td>Iron deficient</td>
<td>325</td>
</tr>
</tbody>
</table>

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carbohydrate reservoir levels. Indeed, Table 1 shows that the Suc pool in the vacuole and the starch content in the amyloplasts increased during the nondividing phases of growth and decreased between cell generations. Furthermore, electron micrographs indicated that starch grains accumulated considerably within amyloplasts whenever growth ceased (not shown).

Comparison of the increase in cell number between iron-deficient and control cultures (Fig. 1a) indicates first that iron deprivation considerably decreased the initial growth rates of the cells. Indeed, in the absence of iron, the cell number doubling time was much higher than that observed with sycamore cells grown in liquid medium containing iron (approximately 100 versus approximately 48 h). Second, iron deprivation limited the maximum density of the cells: After 1.2 to 1.3 generations (versus 2.5–2.7 in the control), the growth rate of iron-deficient cells decreased, leading to a short stationary phase that was followed by a decline in cell viability. After 7 d of iron starvation, when growth had ceased, the total iron content averaged 55 ± 5 ng/10^6 cells, compared with 140 ± 15 ng/10^6 cells for control cells (mean ± se for five experiments). Addition of iron 1 d after growth had ceased caused a large increase in the cell number to the level of normal stationary phase (not shown). Interestingly, during the exponential phase of growth, and in contrast with what was observed with normal cells, the wet weight on a cell unit basis did not decrease and even increased noticeably (Fig. 2a), as did cell protein and polar lipid contents (Table I). Taken together, these observations strongly suggest that iron-deficient cells remained swollen throughout their growth. This is why, although the cell number doubling time had increased, the evolution with time of the wet weight for iron-deficient cell culture was fairly similar to that of normal cells (Fig. 1b). Likewise, during the exponential phase of growth, the cell dry weight did not exhibit any noticeable variation (Fig. 2b) and, for example, the level of intracellular carbohydrate reservoirs (starch and Suc) remained almost unchanged in iron-deficient cells after 3 d of culture (Table I).

One of the most important differences between the growth of control cells and iron-deficient cells was that, when the growth had ceased, the absence of iron in the medium led to a rapid cell death (Fig. 1a). In the absence of iron, we observed a sudden decline in all the physiological parameters about 5 d after the end of the exponential phase of growth. Such a result was not expected because deprivation of other nutrients including copper (Bligny and Douce, 1977) and phosphate (Rédieille et al., 1984) did not lead to sycamore cell death. The reason for the decline in cell viability is unclear. It is possible that, after each cell division, severe iron deficiency brings about large decreases in the iron-containing constituents of the mitochondrial respiratory chain in sycamore mitochondria, i.e. of the Cyt b path capacity, until ATP delivery to the cytosol becomes a limiting factor for general cell metabolism. In other words, after a long period of iron starvation, ATP production in the mitochondria could not satisfy the extramitochondrial ATP requirement. We decided, therefore, to investigate the changes in O2 consumption of sycamore cells during a prolonged period of iron deprivation.

Effect of Iron Deprivation on Plant Cell Respiration

The rate of respiration (Fig. 3. V0) of normal cells expressed on a cell unit basis increased during the lag phase and then declined progressively back to its original value during the exponential phase of growth. Respiration could be decreased by inhibiting ATP production with oligomycin (V0L), a potent inhibitor of the mitochondrial ATPase complex; or increased with an uncoupler such as FCCP (VFCP) (Fig. 3). Interestingly, the uncoupled rate of O2 consumption per cell paralleled the rate of respiration without uncoupler. Moreover, the intracellular level of two specific mitochondrial markers, fumarase activity and cardiolipin (Bligny and Douce, 1980), also exhibited the same variations as the uncoupled respiration rate (Fig. 4). The simplest interpretation of these correlations is that the fluctuation of the O2 uptake rate per cell was not due to intrinsic changes in the mitochondria themselves, but rather to slight variations in the number of mitochondria per cell (or the total inner membrane area in a cell; for an explanation, see Journet et al., 1986), which were tightly related to the cell size fluctuations.

Such an adjustment of the number of mitochondria with the cell volume seemed also to occur in cells grown in the absence of iron. Indeed, during the first 2 to 3 d of culture, when cells enlarged considerably, the respiration rates with

![Figure 3. Influence of iron deficiency on sycamore cell respiration. Cells were regularly taken from control (+Fe) or from iron-deficient (−Fe) cultures during growth and introduced into an O2-electrode chamber. O2 consumption was recorded at 25°C on 1.5 × 10^6 cells in suspension in 1 ml of their culture medium. V0, basal respiratory rate; V0L, and VFCP, respiratory rates in the presence of 40 μg of oligomycin or 1.5 μM FCCP (final concentrations), respectively. O2 uptake rates are expressed in nmol O2 consumed/min per million cells. Data presented here are from one representative experiment that has been reproduced six times with similar results. L, D, and S, Lag, dividing, and stationary phases of cell growth, respectively.](https://www.plantphysiol.org)
or without uncoupler (\(V_o\) and \(V_{\text{FCCP}}\)) expressed on a cell unit basis (Fig. 3), as well as the intracellular levels of fumarase activity and cardiolipin (Fig. 4), increased in the same proportions as in the case of normal cells. Thus, iron deprivation did not prevent the increase in the number of mitochondria per cell during the lag phase of growth. However, we observed that, during the dividing phase, the respiration rate (\(V_o\)) and the levels of the mitochondrial markers per cell remained roughly stable or decreased to lesser extents when compared with the controls, in accordance with the fact that iron-deficient cells remained swollen during their growth. A crucial difference between the respiration of control and iron-deficient cells was that, when mitosis began, the absence of iron led to a steady decline in the uncoupled rate of \(O_2\) consumption, to approach the usual \(O_2\) uptake rate after 7 d of iron deprivation (i.e., when cells ceased to divide). From this point the mitochondria worked in vivo at full capacity and the respiration rate, which was no more stimulated by FCCP, became progressively insensitive to oligomycin and declined abruptly. Interestingly, \(^{31}\)P NMR analysis of cell perchloric acid extracts have indicated that, on a cell unit basis, the absolute concentration of nucleotides did not vary throughout the steady decline in the uncoupled rate of \(O_2\) consumption, but collapsed after 7 to 8 d of iron deficiency (not shown), i.e., when the usual respiration rate was decreasing.

As a whole, these results strongly suggest that iron deprivation led to a progressive impairment of the total respiratory chain capacity in mitochondria, which remained without any consequence on the overall rate of general metabolism as long as ATP delivery by mitochondria was unaffected. But after 7 d of iron deficiency, it is likely that mitochondria became inefficient in producing ATP to keep pace with the cell metabolism. Such a critical situation may contribute to the subsequent cell death. To verify the putative impairment of the total respiratory chain capacity, experiments were undertaken to test whether iron deficiency led to differences in the relative and absolute concentrations of iron-containing electron transport components and whether the oxidative properties of isolated mitochondria were affected.

**Iron-Containing Components of Sycamore Mitochondria**

After 7 d of iron starvation, when growth had ceased, the total iron content of mitochondria averaged 260 ± 40 ng/mg protein, compared with 550 ± 10 ng/mg protein for control mitochondria (mean ± SE for five experiments). This suggests that iron deficiency could lead to a decrease in the concentration of Cys and Fe-S centers.

The difference spectra of normal mitochondria shown in Figure 5 are typical of plant mitochondria when examined at liquid nitrogen temperature. Cyt aa3 has a peak at 599 nm. The \(\alpha\) band of Cyt \(c\) appears at 549 nm. The shoulder at 560

![Figure 4](image_url)

**Figure 4.** Intracellular levels of cardiolipin and fumarase activity during the culture of sycamore cells in the presence or absence of iron. Cell-suspension samples were regularly harvested from control (+Fe) or iron-devoid (-Fe) cultures to measure the intracellular levels of two mitochondrial markers, cardiolipin and fumarase activity, following the procedures described in "Materials and Methods." Cardiolipin contents are expressed in \(\mu\)g per million cells; values are means ± SE for two experiments. Fumarase activities were measured at 240 nm and expressed in nmol fumarate formed/min per million cells; values are means ± SE for four experiments. L, D, and S, Lag, dividing, and stationary phases of cell growth, respectively. Note the sharp decrease in fumarase activity in the 7 and 10 d of iron starvation, whereas at the same time the cell cardiolipin content (reflecting the total mitochondrial inner membrane area) remains constant.

![Figure 5](image_url)

**Figure 5.** Influence of iron deficiency on mitochondrial Cyt content. Mitochondria were purified from 7-d-old control (+Fe) or iron-deficient (-Fe) sycamore cells. Cys were analyzed as described in "Materials and Methods." Difference spectra, dithionite-reduced minus aerobic mitochondria suspended in the electrode medium (3.4 mg protein/mL), were recorded at 77 K. Mitochondrial Cys content (mean values ± SE for three experiments) are expressed in nmol/mg of mitochondrial protein.
nm representing the Cyt b complex in the room-temperature difference spectrum (not shown) has been split into three peaks absorbing at 562, 557, and 553 nm when examined at \(-196^\circ\text{C}\). In the Soret region, Cyt a\(_2\) shows a typical double peak at 444 and 437 nm and the \(b + c\)-type Cytbs give a common peak at 423 nm. These observations are in good agreement with Lance and Bonner (1968). In the case of iron-deficient mitochondria, we observed a striking reduction of all the Cyt peaks. Expressed in terms of nmol/mg of mitochondrial protein, the concentrations of all the Cysts including that of Cyt a\(_2\) were much lower in the iron-deficient mitochondria—less than one-half to one-third of the normal amounts (Fig. 5).

Figure 6 shows EPR spectra of mitochondria from sycamore cells following succinate + pyruvate + dithionite reduction (see Brouquisse et al., 1986). The components seen consisted of a number of Fd-type centers in the reduced state. Following the nomenclature of Ohnishi and Salemo (1978), the most unambiguously resolved components in these spectra were attributed to Fe-S centers of complex I (N.1b, apparent \(g_{xyz} = 1.93, 1.93, 2.017\); N.2, apparent \(g_{xyz} = 1.92, 1.92, 2.05\); N.3, apparent \(g_{xyz} = 1.88, 2.03\)) and complex II (S.1, apparent \(g_{xyz} = 1.92, 2.025\)). It should be noted in this context that attempts to characterize centers S.2 and N.4 were unsuccessful. At higher temperatures (above 25 K), all the features corresponding to 4 Fe-S centers disappeared, especially the peak at \(g_x = 2.05\) (N.2 center), together with a peak at \(g_x = 2.03\) (N.3 center). In the spectra of oxidized mitochondria obtained at 16 K, an intense and nearly isotropic EPR signal was seen at \(g = 2.014\) with a low-field maximum of \(g = 2.02\), which was assigned to center S.3 of succinate dehydrogenase (Brouquisse et al., 1986). A second signal with a peak at a \(g\) value of approximately 2.03 was also present, causing an apparent broadening of the \(g = 2.02\) (center S.3) peak. We observed that, as the temperature was raised above 18 K, the \(g = 2.02\) peak center S.3 diminished rapidly, causing the feature centered at \(g = 2.03\) to become much more prominent. This second signal was assigned to the matrix Fe-S protein aconitase (see Ruzicka and Beinert, 1978; Brouquisse et al., 1987).

The absolute concentration of Fe-S clusters in plant mitochondria cannot readily be determined by EPR alone. However, differences in relative concentration of specific Fe-S clusters in control compared with iron-deficient mitochondrial preparations can be measured. Thus, it is obvious that iron deprivation led to a significant decrease in the concentration of all Fe-S clusters studied, including EPR signals deriving from oxidized mitochondria. For example, the concentrations of clusters S.1 and N.1b were at least 70% lower in mitochondrial preparations from iron-deficient cells than from controls. However, EPR spectra of iron-deficient sycamore mitochondria exhibit some particular features when compared with controls. Indeed, Figure 6 shows that the signals of all classical Fe-S centers around \(g = 2.00\) and \(g = 1.92\) were considerably distorted, especially when the temperature was raised above 18 K. Furthermore, an atypical signal at \(g = 2.045\) (Fig. 6, X), which increased with the temperature, appeared in both reduced and oxidized preparations. This indicates the presence of at least one unknown component in iron-deficient sycamore mitochondria.

**Effect of Iron Deprivation on the Oxidative Properties of Intact Mitochondria Isolated from Sycamore Cells**

As previously shown by Journet et al. (1986), a feature of mitochondria purified from sycamore cells cultivated in a nutrient medium containing iron was their very high rate of \(O_2\) consumption with tricarboxylic acid cycle substrates and external NADH. A comparison between the oxidative properties of iron and iron-deficient mitochondria made at the end of the exponential phase of growth (Table II) revealed a major difference: on a protein basis, the rate of succinate-dependent \(O_2\) uptake in state 3 (the electrons are injected at the level of complex II of the respiratory chain) declined considerably in iron-deficient mitochondria when compared with control mitochondria. In marked contrast, the rates of external NADH-dependent \(O_2\) uptake in state 3 (the electrons are injected at the level of complex III of the respiratory chain via the ubiquinone pool) (Douce and Neuburger, 1989) and cyanide-sensitive Cyt c-dependent \(O_2\) uptake (the electrons are injected at the level of complex IV of the respiratory chain) were about the same for iron and iron-deficient mi-
The mitochondria from normal cells exhibited a dense matrix period of iron starvation on the mitochondria because all of major impairment in the functioning of complex I. Most of the cristae oriented sacs and folds. In marked contrast, after extensive translucent cristae appearing as irregularly our attempts to isolate intact mitochondria from cells harb-

mitochondria oxidizing either external NADH or malate deficiency, mitochondria displayed a more dilute matrix and less cristae than normal mitochondria. On the other hand, the activity of aconitase, a soluble matrix enzyme that contains a iron-sulfur centers (S1 [paramagnetic in the reduced form]; 53 [paramagnetic in the oxidized form]) of complex I1.

CONCLUSIONS

These results demonstrate that incubation of sycamore cells in flasks containing iron-free culture medium triggers the following cascade of reactions. (a) The cell number doubling time increases considerably. (b) During the exponential phase of growth the uncoupled rate of O2 consumption declines progressively, whereas the normal O2 consumption rate remains constant. (c) When the uncoupled O2 uptake rate approaches the normal rate of O2 consumption, the cells stop dividing and begin to collapse. The progressive decline in the uncoupled rate of O2 consumption was not attributable to a progressive diminution of the number of mitochondria per cell because during this period the total intracellular cardiolipin, a specific marker of inner mitochondrial membrane, remained constant. In fact, it is very likely that decreases in the iron-sulfur centers (S1 [paramagnetic in the reduced form]; S3 [paramagnetic in the oxidized form]) of complex II (the segment of the respiratory chain responsible for electron transfer from succinate to ubiquinone), appear to cause a decrease in the cell maximal O2 consumption for the two following major reasons. (a) The marked diminution of the mitochondria cristae seem to vary in proportion to respiratory enzymes (Brosem et al., 1963), the marked decline in the Cyts and Fe-S cluster content of iron-deficient mitochondria should be accompanied by a regression of the cristae if the respiratory chain constituents are not incorporated into the inner mitochondrial membrane when they lack their heme or Fe-S prosthetic groups.

Table II. Effects of iron deficiency on oxidative properties of isolated mitochondria

<table>
<thead>
<tr>
<th>Substrates</th>
<th>O2 Consumption</th>
<th>RCR</th>
<th>ADP:O</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ Fe</td>
<td>− Fe</td>
<td>+ Fe</td>
</tr>
<tr>
<td>Pyruvate + malate + α-ketoglutarate</td>
<td>170</td>
<td>160</td>
<td>1.8</td>
</tr>
<tr>
<td>NADH</td>
<td>245</td>
<td>360</td>
<td>2.0</td>
</tr>
<tr>
<td>Cyt c/ascorbate</td>
<td>770</td>
<td>850</td>
<td>1.6</td>
</tr>
<tr>
<td>Succinate</td>
<td>190</td>
<td>90</td>
<td>1.0</td>
</tr>
<tr>
<td>Citrate</td>
<td>33</td>
<td>17</td>
<td>1.6</td>
</tr>
<tr>
<td>Isocitrate</td>
<td>72</td>
<td>67</td>
<td>1.6</td>
</tr>
</tbody>
</table>

Table II. Effects of iron deficiency on oxidative properties of isolated mitochondria

Mitochondria were purified from 7-d-old control (+Fe) or iron-deficient (−Fe) cells harvested at the end of the dividing phase of growth. Isolated mitochondria (100–300 μg of protein) were suspended in 1 mL (final volume) of electrode medium and introduced in an O2-electrode chamber. O2 consumption was recorded at 25°C, in the presence of various substrates and appropriate cofactors (see "Materials and Methods"). State IV and state III (in the presence of 200 μM ADP) were measured. Maximal values of O2 consumption presented here are given in nmol O2 consumed min−1 mg−1 of protein. ADP:O ratios along with respiratory control ratios (RCR) (state III:state IV) were calculated for each substrate. Data presented here are from one representative experiment that has been reproduced six times with similar results.
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concentration of all the mitochondrial Cytos as well as iron-
sulfur centers of complex I (the segment of the respiratory
chain responsible for electron transfer from internal NADH
to ubiquinone) induced by iron deprivation did not affect the
maximal O2 consumption rate by the mitochondria. (b) The
striking reduction of the iron-sulfur centers S1 and S3 asso-
ciated with complex II observed 7 d after iron deprivation
led to a dramatic decrease in the maximal rate of succinate
oxidation by isolated mitochondria. Parenthetically, such a
result suggests, in agreement with previous data (Bligny and
Douce, 1977), that Cytos are present in large excess in plant
mitochondria, contrary to mammal mitochondria, for exam-
ple (Maguire et al., 1982). The same principle holds true for
the iron-sulfur centers of complex I.

In a very interesting article, Maguire et al. (1982) also
reported that in complex II from skeletal muscle mitochon-
dria, Fe-S clusters S1 and S3 were considerably decreased by
iron deficiency and that these losses of Fe-S clusters caused
a marked decrease in succinate dehydrogenase activity. It is
clear from our results that the tricarboxylic acid cycle func-
tioning should be deeply affected after several days of iron
starvation owing to the failure of complex II to oxidize
succinate at a sufficient rate for physiological maintenance.
In support of this suggestion, preliminary experiments carried
out in our laboratory indicate that the intracellular concen-
tration of succinate increased considerably after 7 d of iron
depprivation, i.e. when the rate of O2 consumption became
insensitive to FCCP.

We also should point out that the decrease in the Fe-S
cluster of aconitase in iron-deficient mitochondria led to an
impairment of citrate oxidation in isolated mitochondria. This
observation suggests that aconitase is probably not present
in large excess in the matrix space. Again, such a situation
should affect the rate of tricarboxylic acid functioning, favor-
ing the accumulation of citrate.

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