Is the availability of substrate for the tricarboxylic acid cycle a limiting factor for uncoupled respiration in sycamore (*Acer pseudoplatanus*) cells?

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Protoplasts obtained from sycamore (*Acer pseudoplatanus*) cell suspensions were found to be highly intact and to retain a high rate of O_2 consumption. If the protoplasts were taken up and expelled through a fine nylon mesh, all the protoplasts were ruptured, leaving the fragile amyloplasts largely intact. Distribution of enzymes of glycolysis in plastids and soluble phase of sycamore protoplasts indicated that the absolute maximum activity for each glycolytic enzyme under optimum conditions exceeded the estimates of the maximal rate at which sycamore cells oxidize triose phosphate. Passage of protoplasts through the fine nylon mesh produced a 3–5-fold decrease in O_2 consumption. However, addition of saturating amounts of respiratory substrates and ADP restored an O_2 consumption equal to that observed with uncoupled intact protoplasts. Taken together, these results demonstrated that neither the overall capacity of the glycolytic enzymes in sycamore cells nor the availability of respiratory substrates for the mitochondria is ultimately responsible for determining the rate of uncoupled respiration in sycamore cells.

INTRODUCTION

The mechanism of regulation of cellular respiration with its accompanying phosphorylation reactions is of particular physiological importance. The flow of substrates such as P_i , nucleotides and tricarboxylic acid-cycle substrates across the mitochondrial membrane during respiration is highly controlled, and probably none of the metabolically important substrates, except CO_2 and O_2 , is able to escape the stringent control exerted by the various specific carriers (P_i , adenine nucleotide, pyruvate and dicarboxylate translocators). At a given temperature the rate at which a cell respire can therefore be theoretically limited by any of a number of factors: (a) availability of substrate for the tricarboxylic acid cycle; (b) the overall capacity of the tricarboxylic acid-cycle enzymes; (c) availability of P_i or of ADP for oxidative phosphorylation; and (d) the overall capacity of the respiratory chain (Douce, 1985). In fact, little attention has been paid to the control of O_2 consumption rate by plant cells. In a previous publication, we showed that, in physiological situations, the respiration rate of sycamore (*Acer pseudoplatanus*) cells is not limited by the quantity of P_i supplied to the mitochondria (Rébeillé *et al.*, 1984). In the present paper we demonstrate that neither the overall capacity of the glycolytic enzymes (cytosolic and plastidic) in a cell nor the availability of substrate for the tricarboxylic acid cycle is ultimately responsible for determining the rate of *uncoupled* respiration in sycamore cells.

MATERIAL AND METHODS

Biological material

The strain of sycamore (maple; *Acer pseudoplatanus* L.) used in the present study was generously provided by Professor J. Guern, Department of Plant Cell Physiology, University of Paris, Paris, France. The basic nutrient medium supplying sucrose as growth substrate was prepared as described by Bligny (1977). Cell suspensions were maintained in exponential growth by frequent subculture. The culture medium was kept at a volume of 0.2 litre, and stirred continuously with a pair of impellers at 60 rev./min. The temperature of the growth culture was 25 °C. Since big starch grains that exhibit a high sedimentation coefficient tear through the amyloplast envelope at supra-optimal *g*-forces, it is necessary to decrease the size of the starch grains before undertaking cell fractionation. To this end, cells were transferred to a sucrose-free medium. Under these conditions, the endogenous starch decreased to 30% of the control value within 24 h (depleted-starch cells) without affecting the maximal rate of O_2 consumption (Rébeillé *et al.*, 1984).

Preparation of protoplasts

Cells (20 g) were suspended in 200 ml of their culture medium containing 0.5 M-mannitol (final osmolarity 0.55 osM), 1% (w/v) cellulase (Onozuka RS; Yakult Pharmaceutical Co, Nishinomya, Japan) and 0.1% (w/v) pectolyase Y-23 (Seishi Pharmaceutical Co, Tokyo, Japan), adjusted to pH 5.7. The cells were incubated with

Abbreviations used: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; PFK, phosphofructokinase; PFP, pyrophosphate:fructose-6-phosphate phosphotransferase; TMPD, tetramethyl-*p*-phenylenediamine.

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Table 1 Subcellular localization of fumarase (mitochondrial marker), ADP-glucose pyrophosphorylase (plastidial marker) and phosphoenolpyruvate carboxylase (cytosolic marker) in mechanically ruptured sycamore-cell protoplasts followed by centrifugation (12000 *g* for 10 min)

Preparation of intact and broken protoplasts, and centrifugation of intact organelles, were carried out as described in the Materials and methods section. These data are from a representative experiment and have been reproduced three times.

Enzyme	Total extract (nmol/min per 10 ⁶ cells)	Distribution (% of total activity)	
		supernatant	pellet
Fumarase	26	8	99
ADP-glucose pyrophosphorylase	2.5	19	82
Phosphoenolpyruvate carboxylase	6.4	89	9

constant shaking (20 cycles/min) at 25 °C. Digestion of young cells at 25 °C for 1 h resulted in a final yield of protoplasts of near 100%. Shaking prevented cell sedimentation. However, vigorous shaking at any stage of the digestion or purification of sycamore protoplasts had a deleterious effect on the subsequent rate of respiration. After digestion the suspension was filtered through one layer of Miracloth (Krantex, Alfortville, France) that retained any undigested cell aggregate, and protoplasts were collected by centrifuging at 150 *g* for 10 min and then washed twice with 50 ml of culture medium containing 0.5 M-mannitol (suspension medium). Protoplasts were stored in suspension medium and were normally used within 2–3 h.

Gentle rupture of protoplasts and separation of the organelles from the cytosolic fraction

Since sycamore-cell protoplasts have an average diameter of 20–30 μm , a rapid and effective procedure for the gentle rupture of intact protoplasts (i.e. for stripping the cell membrane) is to pass depleted-starch protoplasts through a fine nylon mesh (20 μm pore size) affixed to the cut end of a 25 ml disposable syringe (Leegood & Walker, 1983). Thus protoplasts (equivalent to 20×10^6 cells) were pelleted after dilution with suspending medium and resuspended in about 5 ml of a basic medium containing 0.5 M-mannitol, 0.1% (w/v) bovine serum albumin, 1% (w/v) insoluble polyvinylpyrrolidone ($M_r \sim 25000$; Serva) and 5 mM-phosphate buffer, pH 7.4; this last addition was chosen because it corresponds to the phosphate concentration and the pH of the cytosolic compartment previously determined by ³¹P n.m.r. studies (Martin *et al.*, 1982). If the protoplasts are taken up and expelled through the 20 μm nylon mesh twice, they are completely ruptured (broken protoplasts). In order to check that none of the soluble markers was released from plastids and mitochondria after this treatment, we subjected the broken-protoplast fraction to centrifugation to yield a pellet largely free of cytosol (Table 1). In order to prevent starch grains from tearing through the plastid envelope, the centrifugal *g*-force was gradually increased by using an automatic rate controller (Sorvall) and centrifugation was carried out in three steps (100 *g* for 5 min, 500 *g* for 5 min and 12000 *g* for 10 min; Sorvall rotor SS 34). Each supernatant was centrifuged in a new tube, and the three successive pellets were combined together (particulate fraction). It can be clearly seen (Table 1) that negligible amounts of the total fumarase (mitochondrial marker) and ADP-glucose pyrophosphorylase (plastidial marker)

were found in the final supernatant (cytosolic fraction). Consequently this procedure ruptures all the protoplasts (broken protoplasts) leaving the fragile plastids containing big starch grains and mitochondria largely intact.

Isolation of mitochondria

Mitochondria from sycamore protoplasts were isolated and purified as described by Nishimura *et al.* (1982) by using discontinuous Percoll gradients. The mitochondria subsequently concentrated by differential centrifugation were better than 95% intact as judged by their impermeability to cytochrome *c* (Neubergur *et al.*, 1982).

O₂ consumption

Sycamore-cell respiration was measured at 25 °C in their culture medium. Protoplast respiration was measured at 25 °C in basic medium with the stirrer is set at its lowest rate consistent with an adequate response of the electrode. Breakage of the fragile protoplasts is thus minimized (Leegood & Walker, 1983). Broken-protoplast respiration was measured at 25 °C in basic medium (see under 'Gentle rupture of protoplasts . . .') containing 5 mM-MgCl₂. The reaction medium for mitochondrial respiration contained 0.3 M-mannitol, 10 mM-KCl, 5 mM-MgCl₂, 5 mM-phosphate buffer, pH 7.2, 0.1% (w/v) defatted bovine serum albumin. O₂ uptake was monitored polarographically with a Clark-type oxygen electrode system purchased from Hansatech Ltd., King's Lynn, Norfolk, U.K. The O₂ concentration in the air-saturated medium at 25 °C was taken as 240 μM .

Measurement of enzymic activities

All assays were first performed on blanks to detect any non-specific drift or endogenous substrate or enzyme contamination. They were then optimized with respect to the concentration of each component and to the pH of the reaction mixture. We verified that, under these conditions, activities were linear with respect to time for at least 2 min and were proportional to the amount of either broken protoplasts or protoplast fraction (12000 *g* pellet or 12000 *g* supernatant); the possible inhibition of enzymes by some inclusions was thus investigated. For the reactions that were continuously monitored with a spectrophotometer (Kontron, Uvikon 810), the coupling-enzyme systems were checked not to be rate-limiting. When NAD(P)H was present in the assay, the rates were corrected for any NAD(P)H oxidase activity. Unless

otherwise stated, all enzymes were assayed at 25 °C in 0.5 ml final volume and the buffer used was either 50 mM-Tricine/NaOH (for pH values above 7.4) or 50 mM-Mops/NaOH (for pH values under 7.4). References to the procedures are given, together with any features of the reaction mixtures that differed from those in the references. A unit of enzymic activity is defined here as the amount of enzyme that catalyses the transformation of 1 μ mol of substrate/min.

Phosphorylase (EC 2.4.1.1) was assayed as P_i -dependent glucose 1-phosphate-forming activity (Preiss *et al.*, 1980); the incubation mixture contained 10 mM-potassium phosphate, pH 7.0, 2 mM- $MgCl_2$ and amylopectin (2 mg·ml⁻¹; from potato; Sigma). ADP-glucose pyrophosphorylase (EC 2.7.7.27) (Espada, 1966) was assayed at pH 8.0 in 1 mM- PP_i /5 mM-NaF/500 μ M-ADP-glucose/5 mM-glycerate 3-phosphate/5 mM- $MgCl_2$ /2 mM-dithiothreitol/10 μ M-glucose 1,6-bisphosphate/500 μ M-NADP phosphoglucomutase (0.8 unit)/glucose-6-phosphate dehydrogenase (0.7 unit). For hexokinase (EC 2.7.1.1; Miernyk & Dennis, 1983) assay the reaction was coupled to the enzymic assay of ADP. Phosphoglucomutase (EC 2.7.5.1; Simcox *et al.*, 1977) was assayed at pH 7.6 in 5 mM- $MgCl_2$ /2 mM-dithiothreitol/10 μ M-glucose 1,6-bisphosphate/2 mM-glucose 1-phosphate/500 μ M-NADP/glucose-6-phosphate dehydrogenase (1 unit). Phosphoglucose isomerase (EC 5.3.1.9; Simcox *et al.*, 1977) was assayed in 5 mM- $MgCl_2$ /2 mM-fructose 6-phosphate/500 μ M-NADP. Fructose 6-phosphate kinase (PFK; EC 2.7.1.11; Kelly & Latzko, 1977) was assayed at pH 7.9 in 5 mM- $MgCl_2$ /5 mM-KCl/1 mM-ATP/5 mM-fructose 6-phosphate/200 μ M-NADH (no dithiothreitol). Pyrophosphate:fructose-6-phosphate phosphotransferase (PFP; EC 2.7.1.90; Carnal & Black, 1983) was carried out as for the PFK assay, except that (i) the sample was preincubated for 2 min with 2 μ M-fructose 2,6-bisphosphate, (ii) that the reaction was initiated with 1 mM- PP_i instead of ATP, and (iii) that 5 mM-NaF was added in order to inhibit any pyrophosphatase activity, without affecting the PFP activity. Fructose, 1,6-bisphosphate aldolase (EC 4.1.2.13) was assayed as described by Horecker (1975). Triose-phosphate isomerase (EC 5.3.1.1) was assayed as described by Beisenherz (1955) with 4 mM-DL-glyceraldehyde 3-phosphate as substrate. Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12; Bergmeyer, 1974) was assayed at pH 7.8 in 2 mM-EDTA/2 mM- $MgCl_2$ /5 mM-dithiothreitol/200 μ M-NAD(P)H. Phosphoglycerate kinase (EC 2.7.2.3; Scopes, 1975) was assayed in 1.5 mM- $MgCl_2$ /1 mM-ATP/5 mM-glycerate 3-phosphate. Phosphoglycerate mutase (EC 2.7.5.3) activity was assayed in four different ways as reported by Stitt & ap Rees (1979), with minor modifications; the reliability of these methods was carefully assessed. Enolase (EC 4.2.1.11; ap Rees *et al.*, 1975) was assayed as pH 7.6 in 5 mM- $MgCl_2$ /10 mM-KCl/1 mM-ADP/200 μ M-NADH/1 mM-glycerate 2-phosphate. Pyruvate kinase (EC 2.7.1.40; Duggleby & Dennis, 1973) was assayed in 5 mM- $MgCl_2$ /40 mM-KCl/2 mM-dithiothreitol/1 mM-ADP/200 μ M-NADH/1 mM-PEP. Fumarase (EC 4.2.1.2) was assayed as described by Hill & Bradshaw (1969). Phosphoenolpyruvate carboxylase (EC 4.1.1.31; Wong & Davies, 1973) was determined either by spectrophotometric assay coupled with malate dehydrogenase or by measuring the amount of ¹⁴C incorporated into malate from $NaH^{14}CO_3$.

Fig. 1. O_2 consumption by sycamore cells (a) and their protoplasts (b)

Cells harvested from the culture medium (2.5×10^6 cells/ml) and protoplasts (2.5×10^6 protoplasts/ml) were directly introduced in the 1 ml electrode chamber. Preparation of protoplasts was carried out as described in the Materials and methods section. Values along the traces represent the O_2 -uptake rates (nmol of O_2 /min per 10^6 cells or protoplasts). Measurements were made at a temperature of 25 °C. The concentrations given are the final concentrations in the reaction medium.

RESULTS AND DISCUSSION

Fig. 1 indicates that protoplasts from sycamore cells harvested after 5–7 days of subculture exhibited high rates of O_2 uptake (10 nmol/min per 10^6 cells). Stimulation of respiration by FCCP was observed repeatedly when it was present at concentrations of 1 μ M or above. There were slight variations in the amount of stimulation observed in different experiments, but the maximum obtained was usually about 100% above the control (Fig. 1). The marked and prolonged stimulation of endogenous respiration obtainable with FCCP suggests that there must be endogenous substrates available in non-limiting amounts (see Givan & Torrey, 1968). In fact, sycamore cells had a large intracellular sucrose concentration (80 ± 10 mM) and starch content (65 ± 10 mM, expressed as glucose) (Rébeillé *et al.*, 1984). Addition of 100 μ M-KCN gave rise to a strong inhibition of O_2 consumption. The strong response to KCN indicated that the activity of an alternative, non-phosphorylating, cyanide-insensitive electron-transport pathway was very low in sycamore protoplasts. Interestingly, protoplast respiratory activities matched those of the parent cells (Fig. 1). In our opinion, retention of a high rate of respiration is a useful criterion of protoplast integrity.

The complete oxidation of one sucrose molecule

Table 2. Distribution of glycolytic enzymes and phosphorylase in plastids and soluble phase (cytosol) of sycamore-cell protoplasts

Preparation and gentle rupture of protoplasts were carried out as described in the Materials and methods section. These data are from a representative experiment and have been reproduced at least three times. In separate experiments (not shown), we confirmed that sycamore-cell plastids, purified by isopycnic centrifugation in density gradients of Percoll (see Journet & Douce, 1985), possess all the glycolytic enzymes except phosphoglycerate mutase. In contrast, the glycolytic enzymes, except hexokinase, were not detectable in purified mitochondria.

Enzyme	Total extract (nmol/min ⁻¹ per 10 ⁶ cells)	Distribution (% of total activity)	
		Cytosol	Particulate fraction
Hexokinase	16	55	45
Phosphoglucumutase	65	90	10
Phosphoglucose isomerase	155	64	36
Fructose-6-phosphate kinase	13	50	50
PFP	42	100	0
Aldolase	56	80	20
Triose-phosphate isomerase	6800	83	17
Glyceraldehyde-3-phosphate dehydrogenase	800	91	9
Phosphoglycerate kinase	590	89	11
Phosphoglycerate mutase	200	100	0
Enolase	210	84	16
Pyruvate kinase	130	75	25
Phosphorylase	2.9	0	100

requires 12 O₂ (Beevers, 1961), and sycamore protoplasts (or cells) exhibited an uncoupled O₂-uptake rate of 20 nmol/min per 10⁶ cells (Fig. 1). From the knowledge of both these parameters, one can calculate that 1.6 nmol of sucrose/min per 10⁶ cells (or 6.4 nmol of triose phosphate/min per 10⁶ cells) were required in order to sustain the uncoupled respiration rate of sycamore protoplasts. In order to know if the absolute activities of glycolytic enzymes exceed the estimates of the rates at which sycamore cells oxidize carbohydrate in the presence of an uncoupler, experiments were performed to determine the maximum activities of each glycolytic enzyme in the cytosolic and plastidic compartments.

Enzymes of the glycolytic pathway in cytosol and particulate fraction

The distribution of enzymes of glycolysis in the particulate fraction, containing almost all the cell plastids, and soluble phase of sycamore protoplasts is shown in Table 2. This Table demonstrates that the cytosolic fraction from sycamore protoplasts contains all of the enzymes of glycolysis. Quantitative consideration of our data shows that the absolute maximum activity for each glycolytic enzyme under optimum conditions exceeds the estimates of the maximal rate at which sycamore cells oxidize triose phosphate. Although the maximum activity of PFK was 2–10 times lower than that of other glycolytic enzymes, the rate may still be enough to meet the demands for respiratory substrates. Interestingly, the reversible PFP (Carnal & Black, 1979), which is activated by fructose 2,6-bisphosphate (Sabulase & Anderson, 1981), exhibits a maximal activity 3–4 times greater than that of PFK (Table 2). In addition, this enzyme seems to be confined in the cytosolic fraction (Table 2). Consequently it would seem that PFP plays at least as important a role in cytosolic glycolysis of the sycamore cells as does PFK, provided that sufficient concentrations of pyrophosphate are present (Van Schaftingen *et al.*, 1982; Kruger *et al.*, 1983). However,

the exact quantity of carbon flowing through either pathway during the course of respiration remains to be quantitatively established in plant cells. Table 2 also indicates that the particulate fraction containing plastids can catalyse glycolysis sufficiently rapidly to sustain the maximal rate of respiration as far as glycerate 3-phosphate. Further metabolism of the latter via glycolysis would have to involve export (via the phosphate translocator) to the cytoplasm, because phosphoglyceromutase is absent from plastids [see also Stitt & ap Rees (1979) in the case of pea shoot chloroplasts; Journet & Douce (1985) in the case of cauliflower bud plastids].

However, since glycolysis is regulated by the intracellular concentration of various metabolites [for review, see ap Rees (1980) and Turner & Turner (1980)] the possibility was not completely excluded that the uncoupled respiration rate of intact cultured sycamore cells is limited by the quantity of substrate supplied to the mitochondria through glycolysis. In order to solve this problem, experiments were undertaken to test whether the flooding of broken protoplasts with saturating amounts of respiratory substrates, in the presence of ADP, triggers an O₂ uptake higher than that of fully uncoupled intact protoplasts (the majority of uncoupling agents such as FCCP induce an increase in the rate of respiration and the final rate obtained is identical to the rate obtained in the presence of ADP).

Respiratory response of sycamore protoplasts to various substrates

The endogenous respiration of intact protoplasts from sycamore cells was not stimulated by various respiratory substrates such as NADH, malate + pyruvate, separately or in combination (Table 3) at all the pH values tested (5–7.5). It is very likely that the plasma membrane presents an impenetrable barrier to added soluble anions. In contrast with corn root protoplasts (Lin, 1982), there was also no response to added NADH (Table 3; Fig. 2) unless protoplasts were slightly damaged.

Table 3. O₂ consumption by sycamore-cell protoplasts (intact and broken)

O₂ uptake was measured as described in Fig. 2. Preparation of intact and broken protoplasts were carried out as described in the Materials and methods section. Measurements were taken at a temperature of 25 °C. The reagents used were 2 mM-NADH, 15 mM-malate, 2 mM-pyruvate, 1 mM-ADP and 2 μ M-FCCP. The basic reaction medium (see the text and Fig. 2) contained in addition 5 mM-MgCl₂ and 0.2 mM-thiamin pyrophosphate (with pyruvate + malate). These data are from a representative experiment and have been reproduced at least ten times.

Substrates	Oxygen consumption (nmol/min per 10 ⁶ protoplasts)		
	Intact protoplasts		Broken protoplasts
	– FCCP	+ FCCP	
None (control)	9	16	3
NADH	9	16	6
NADH + ADP	9	16	16
Malate + pyruvate	9	16	5
+ ADP	9	16	13
+ NADH	9	16	6
+ NADH + ADP	9	16	16

Fig. 2. Effect of NADH and ADP on O₂ consumption by sycamore-cell protoplasts

(a) Intact protoplasts; (b) broken protoplasts. Intact protoplasts (2.5×10^6 protoplasts/ml) and broken protoplasts (equivalent to 2.5×10^6 protoplasts/ml) were directly introduced in the 1 ml electrode chamber containing 0.5 M-mannitol, 0.1% (w/v) bovine serum albumin, 1% (w/v) insoluble polyvinylpyrrolidone, 5 mM-phosphate buffer, pH 7.4, and 5 mM-MgCl₂. Preparation of intact and broken protoplasts were carried out as described in the Materials and methods section. Values along the traces represent the O₂-uptake rates (nmol of O₂/min per 10⁶

Table 4. O₂ consumption by sycamore-cell mitochondria

Mitochondria were purified and O₂ consumption measured immediately as described in the Materials and methods section. Mitochondria (0.3 mg of protein) were added to 1 ml of electrode medium containing 2 mM-ADP in an O₂ electrode vessel. The appropriate substrate was added and O₂ uptake measured until a steady state was obtained. Substrates used were 2 mM-NADH, 15 mM-malate, 2 mM-pyruvate, 8 mM-ascorbate, 2 mM-TMPD and 2 mM-ADP. With pyruvate and malate as substrates, the electrode medium contained in addition 0.2 mM-thiamin pyrophosphate and 0.2 mM-NAD⁺. These data are from a representative experiment and have been reproduced at least ten times.

Substrates	O ₂ consumption (nmol/min per mg of protein)
Malate + pyruvate	250
NADH	340
Ascorbate – TMPD	360

Passage of protoplasts through the 20 μ m nylon mesh produced a 3–5-fold decrease in O₂ consumption (Fig. 2). Such a decrease was attributable to a massive leakage of various endogenous substrates and cofactors such as ADP, NAD⁺ and Mg²⁺. Addition of 5 mM-MgCl₂ and respiratory substrates (malate + pyruvate) to broken protoplasts in basic medium did stimulate O₂ uptake (Table 3). Addition of ADP (Table 3) or of FCCP (not shown) produced an immediate 2–3-fold increase in O₂ consumption, and the respiration of broken protoplasts approximated that in the intact protoplasts from which they were derived (Table 3). Addition of NADH

protoplasts). Measurements were made at a temperature of 25 °C. The concentrations given are the final concentrations in the reaction medium. Further abbreviation used: cyt. c, cytochrome c.

stimulated further the rate of respiration. However, the final rate obtained never went beyond the uncoupled rate obtained in intact protoplasts. Interestingly, the State-3 rate of O_2 uptake with NADH alone was equal to that observed with uncoupled intact protoplasts (Fig. 2; Table 3). The strong reactivity of a cytoplasmic laccase-type polyphenol oxidase (Bligny & Douce, 1983) with ascorbate plus TMPD (results not shown) prevented us from utilizing this artificial electron donor in sycamore protoplasts. However, since the State-3 rate of O_2 uptake with NADH alone by laccase-free sycamore mitochondria was equal to that with ascorbate plus TMPD as substrates (Table 4), it is clear that the cytochrome chain was saturated with electrons when NADH was oxidized and that the cytochrome *c* oxidase became the rate-limiting step in the presence of ADP or an uncoupler.

Taken together, these results demonstrate that the availability of respiratory substrates for the mitochondria is not responsible for determining the rate of uncoupled respiration in sycamore cells. This supports the conclusion reached by Givan & Torrey (1968) with a greater range of potential respiratory substrates and with a direct comparison with maximum rates of NADH oxidation. It is clear, however, that factors determining the rates of coupled respiration remain to be elucidated.

Concluding remarks

The data taken as a whole leave little doubt that the *uncoupled* respiration rate of intact cultured sycamore cells is not limited by the flow of substrate (malate, pyruvate and NADH) supplied to the mitochondria through glycolysis. The two major arguments in favour of this conclusion are: (a) enzymes capable of catalysing each of the glycolytic steps are present at sufficient maximal activity to sustain the maximal rate of O_2 consumption and therefore to mediate the measured flux of carbon through the mitochondria; (b) addition of ADP, Mg^{2+} and saturating amounts of substrates (malate, pyruvate and NADH) to broken protoplasts triggers a rapid O_2 consumption, and the maximal respiration thus obtained closely approximates that in uncoupled intact protoplasts. In addition, in sycamore-cell mitochondria, since the rate of O_2 uptake with ascorbate plus TMPD was equal to that of saturating amounts of substrates in the presence of ADP or an uncoupler, we can conclude that the uncoupled rate at which sycamore cells respire is limited by the overall capacity of the respiratory chains.

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