Respiratory Response of Acer pseudoplatanus Cells to Pyruvate and 2,4-Dinitrophenol

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Abstract. The endogenous respiration rate of unstarved cultured cells of Acer pseudoplatanus L. is markedly stimulated by 2,4-dinitrophenol. Pyruvate is also stimulatory but to a lesser degree than dinitrophenol. Exogenously supplied sugars cause no short-term stimulation. Pyruvate does not enhance the elevated rate of O₂ uptake in the presence of dinitrophenol but does cause additional CO₂ evolution. The endogenous concentration of pyruvate is elevated in the presence of dinitrophenol. These observations suggest that the rate of O₂ uptake by the unstarved intact cells is limited by the rate of glycolysis and that rate of glycolysis is regulated by the intracellular concentration of adenine nucleotides or inorganic phosphate. Dinitrophenol stimulation of endogenous respiration is due in part to an indirect acceleration of glycolysis but also to a more direct facilitation of oxidation in the presence of excess mitochondrial substrate.

Despite much recent progress in elucidating the chemical pathways involved in the respiration of higher plants, relatively little information is available concerning the control of rates of oxygen consumption by intact plant cells. Although the respiration rate of many nongreen plant tissues falls off when they are deprived of a carbon source for long periods of time, and the reduced respiration rates resulting from such starvation can often be elevated by supplying appropriate sugars (13), it is nevertheless clear that the respiratory rate of plant cells is frequently not limited by the availability of carbohydrates. For example, in the presence of excess substrate, the respiration rate of many intact cells or of isolated mitochondria can be markedly stimulated by compounds which uncouple oxidative phosphorylation from respiratory electron flow (8).

Although the most direct effect of an uncoupling compound such as 2,4-dinitrophenol (DNP) is to disrupt the normal linkage between electron flow and oxidative phosphorylation (15), a DNP-induced stimulation of O₂ consumption by intact cells need not be wholly the result of removing a constraint upon electron flow in the mitochondrial cytochrome chain. Uncoupling electron flow from the synthesis of ATP not only facilitates electron flow but may also produce alterations in intracellular concentrations of adenine nucleotides and P₁ or may effect a redistribution of these compounds between the intra- and extra-mitochondrial compartments (6). Because increases in ADP or P₁ or decreases in ATP are known to speed up the rate of glycolysis (16), it has been suggested that at least part of the respiratory stimulation evoked by uncoupling agents may stem from additional mitochondrial substrate (pyruvate) which is thereby made available (2). If the amount of pyruvate produced by glycolysis is insufficient to saturate the respiratory capacity of the mitochondria, indirect stimulation of glycolysis brought about by DNP treatment could lead to increased O₂ uptake simply by supplying the mitochondria with additional substrate. If, on the other hand, the mitochondria are normally respiring in the presence of excess substrate, any stimulation of O₂ uptake can be ascribed to a removal of restraint on respiratory electron flow or to the activation of tricarboxylic acid cycle enzymes.

Previous studies on the effects of uncoupling agents have suggested that uncouplers stimulate both glycolysis and respiration in higher plant tissues, just as in yeast cells or animal tissues. Newcomb (17), working on tobacco callus, found that at certain concentrations, DNP led not only to increased O₂ uptake but also to an even greater stimulation of CO₂ evolution; i.e., there was an increase in respiratory quotient (R. Q.). This rise in R. Q. indicated a stimulation of glycolysis proportionally greater than the respiratory increase. The intracellular pyruvate concentration presumably increased to the point where it could not be accommodated by the mitochondrial oxidizing system so that the excess was decarboxylated. This interpretation was corroborated by Beevers (2) and by Bianchetti and Marre (4) who likewise found that DNP treatment caused a rise in R. Q. When R. Q.

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values above 1.0 occurred, these workers were able to detect the formation of acetaldehyde and alcohol. Beever's conclusion that the overall respiratory stimulation elicited by DNP was at least partly due to an increased supply of pyruvate owing to enhancement of the glycolytic flux. To an extent, however, Beever's data left open the question whether the overall respiratory stimulation could be ascribed to increased glycolysis alone. Also, as noted by Turner (19), the possibility was not completely excluded that the aerobic fermentation occurring in the presence of DNP might have been brought about if the rapid respiration of cells on the exterior of the tissue fragments had acted to deprive the interior cells of O2, causing them to switch over to a fermentative metabolism essentially under anaerobiosis.

The following experiments were undertaken to test the respiratory response of cultured *Acer pseudoplatanus* cells to DNP and pyruvate, with the goal of bringing to light some of the general limiting factors regulating oxygen uptake by the cells. Consideration has been given to the question of the extent to which the respiratory response to DNP involves regulation of glycolysis.

**Materials and Methods**

Cultured cells were derived from a strain originally isolated by Lamport (14), from stem tissue of *Acer pseudoplatanus* L., a woody angiosperm. Callus cultures were maintained on a modification of the M6 medium of Torrey and Shigemura (18), containing Na-Fe-ethylenediaminetetraacetate (19 mg/l) in place of FeCl3, and containing 2,4-dichlorophenoxacyetic acid at a concentration of 1 μM. The medium was solidified with 0.8 % Difco Bacto Agar. Liquid suspension cultures were initiated by transferring pieces of callus to liquid M6 medium containing 2,4-dichlorophenoxacyetic acid at 10 μM. These cultures were incubated on a horizontal table shaker, rotary action, operating at 80 revolutions per minute. Liquid cultures were routinely subcultured by 1:11 dilution pipetted into fresh medium every 7 to 10 days.

Cells used for experiments were harvested after 7 to 11 days of subculture, centrifuged at 200 × g for 2 minutes, washed once in approximately 15 volumes of 0.05 μM McIlvaine's citrate-phosphate buffer at pH 4.1 (7), centrifuged again, and resuspended to give a suspension having a packed cell volume equal to 10 to 20 % of the total volume of buffered suspension. Preliminary tests showed that the presence of citrate in the buffer did not appreciably alter the cellular respiration rate in comparison to the rate in 0.1 μM phosphate buffer at pH 5.2.

Uniform aliquots of cell suspension were pipetted into Warburg flasks, followed by a smaller aliquot of substrate or uncoupler. For many experiments it was convenient to pipette 2.5 ml of buffered cell suspension into the main compartment of the flask, followed by 0.5 ml of uncoupler or pyruvate at 6 times the desired concentration. All solutions of pyruvate or DNP were made up in 0.05 μM McIlvaine's buffer at pH 4.1.

For measurements of O2 uptake, 0.2 ml of 10 % (w/v) KOH were pipetted into the center well containing a fluted square of Whatman No. 1 filter paper. When CO2 evolution was to be measured by the "2-flask" method, 0.2 ml of buffer rather than KOH were added to the center well in 1 set of flasks. Cells received approximately 40 minutes pretreatment with the experimental chemicals prior to the time the stopcocks were closed and manometric readings begun. Manometers were usually read every 20 minutes for a period of 2 hours. Incubation temperature was 25 °C. Shaking was at 120 cycles per minute with an amplitude of 2.5 cm. Unless otherwise stated, the gas phase was air.

In experiments where endogenous pyruvate concentration was measured, 2.4 ml of suspension in each Warburg flask was deproteinized by the addition of 0.6 ml of 30 % (w/v) perchloric acid from the sidearm. After shaking for 10 to 15 minutes at 25 °C, the deproteinized suspension from duplicate flasks was combined and cooled in an ice bath for 20 minutes and then centrifuged at 1000 × g for 5 minutes. The pellet was then reextracted with 6 ml 6 % (w/v) perchloric acid at 0 °C for 20 minutes. After centrifuging as above, the 2 extracts were combined and neutralized to a pH of approximately 4.5 to 5.5 with 70 % (w/v) K2CO3. After allowing the neutralized extracts to stand at 0 °C for at least 10 minutes, the precipitated potassium perchlorate was removed by centrifugation. The perchlorate-free extracts were analyzed for pyruvate using lactate dehydrogenase according to Bergmeyer (3). DPNH oxidation was monitored by means of a double monochromator spectrophotometer with the measuring beam at 340 nm and the reference beam at 380 nm. Calibration was achieved by adding known amounts of pyruvate to the extract after the pyruvate initially present in the extract had been converted to lactate in the presence of lactate dehydrogenase.

**Results and Discussion**

The respiratory rate of the buffered cell suspension was not increased by hexose substrates or by sucrose over the 2 hour measuring period; however, a respiratory decline which appeared after 5 to 7 hours in buffer could be partially counteracted by glucose, sucrose, and mannose. The lack of short-term response to exogenous sugar suggested that cells taken directly from culture medium were already saturated with endogenous carbohydrate substrate, so that additional amounts of sugar would not raise the respiration rate above its
endogenous level. The possibility that sluggish penetration of the cells by sugars might account for their lack of effect has not been rigorously excluded. However, this seems rather an unlikely explanation. The marked and prolonged stimulation of endogenous respiration obtainable with dinitrophenol (see below) suggests that there must be endogenous substrates available in nonlimiting amounts. The cells contain starch which may support the endogenous respiration. In any event the cells possess a large pool of glucose-6-P indicating an abundance of endogenous substrates which constantly replenishes the pool of hexosemonophosphates as these compounds are metabolized (unpublished observations).

A respiratory response to dinitrophenol was observed repeatedly at concentrations of 1 μM or greater. With increasing DNP concentration from 1 to 10 μM, there was increasing stimulation of O₂ uptake (fig 1). Higher concentrations of DNP were inhibitory. The concentration dependence of the respiratory response to dinitrophenol is very striking and confirms that previously described (2,17). There was some variation in the amount of stimulation observed in different experiments, but the maximum obtained was usually about 60 to 80% above the control. The response to DNP indicated that respiration was regulated at some point by the prevailing intracellular concentrations of adenine nucleotides or Pi.

Sodium pyruvate at 10 mM increased the rate of O₂ uptake, and this was true irrespective of whether exogenous sugar was also supplied to the cells. Pyruvate at this concentration produced a stimulation which was, however, characteristically less than that which could be obtained with DNP. O₂ uptake was usually stimulated 25 to 50% above the rate of controls by 10 mM sodium pyruvate. Higher pyruvate concentrations did not lead to any further increase in respiration rate, but were, in fact, inhibitory (fig 2). Consequently, the respiration rate occurring in 10 mM pyruvate represents the maximum rate attained with pyruvate present in excess (in the absence of uncoupler). Since an increase in respiration rate was always observed when the cells were supplied with pyruvate (in the absence of DNP), it seemed clear that when only endogenous substrate was available, the mitochondria were not operating in the presence of excess intracellular pyruvate. This is similar to the situation in *Avena* coleoptile tissue (5), but contrasts with that in bakers' yeast (10).

In view of the above findings, it seemed likely that the rate of glycolysis might be an important factor controlling the rate of O₂ uptake by intact *Acer* cells. Likewise it seemed probable that any respiratory response to dinitrophenol might be due in part to acceleration of glycolytic flux which would supply the mitochondria with additional substrate. In order to investigate this possibility in more detail, we conducted further experiments in which DNP at concentrations of 5 μM and 10 μM was supplied to *Acer* cells in the presence and absence of 10 mM sodium pyruvate. Figures 3a and 3b show the results of 2 of these experiments. The 2 DNP concentrations employed were the one previously found to elicit maximum stimulation and a lower concentration which produced only moderate enhancement (see fig 1). It was found that although pyruvate or DNP supplied individually to the cells accelerated the respiration rate, the re-

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**Fig. 1.** Effect of different concentrations of dinitrophenol on O₂ uptake. Experimental period 2 hour. Control = 98 μl O₂/flask/hr.

**Fig. 2.** Effect of different concentrations of sodium pyruvate on O₂ uptake. Experimental period 1 hour. Control = 100 μl/flask/hr.
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\[ \text{O}_2 \text{ uptake} \quad \text{O}_2 \text{ evolution} \]

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<tr>
<th>Treatment</th>
<th>O2 Uptake (mL/hr)</th>
<th>CO2 Evolution (mL/hr)</th>
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<tr>
<td>Control</td>
<td>100</td>
<td>150</td>
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<tr>
<td>No pyruvate</td>
<td>200</td>
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<td>1 X 10^-2M DNP</td>
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<td>1 X 10^-6M DNP + pyruvate</td>
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<td>DNP</td>
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<td>DNP + pyruvate</td>
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Fig. 3. Effect of 10 mM sodium pyruvate on O2 uptake and CO2 evolution in the presence of 5 \( \mu \text{M} \) and 10 \( \mu \text{M} \) dinitrophenol. Experimental period 2 hours. Figures 3a (top) and 3b (bottom) based on 2 separate experiments done on different days.

response to 10 \( \mu \text{M} \) DNP clearly exceeded the response to pyruvate alone (fig 3b) whereas the respiratory responses to 5 \( \mu \text{M} \) DNP and 10 mM pyruvate were roughly equivalent (fig 3a). With either DNP concentration, the rate of O2 uptake was not further enhanced by the simultaneous addition of pyruvate along with the uncoupler (figs 3a and 3b).

These latter observations suggest further conclusions. In the absence of DNP, the rate of O2 uptake can be increased by pyruvate, but in the presence of DNP, pyruvate has no stimulatory effect on O2 consumption. It therefore appears that in the absence of DNP the rate of pyruvate formation by the glycolytic reaction pathway of the cells (and thus the supply of substrate for mitochondrial oxidation) limits the rate of O2 uptake. On the other hand, pyruvate production is no longer limiting in the presence of DNP. Therefore, the uncoupling compound must accelerate the glycolytic flux to the point where the mito-

chondria are receiving an excess of substrate, so that further amounts of pyruvate supplied exogenously have no effect. It should be emphasized that these data demonstrate DNP-induced acceleration of glycolysis under conditions where the R. Q. does not rise significantly above 1.0. This fact, together with the fact that the cells are in suspension and not subject to the existence of diffusion barriers, makes it possible to dispose of the above mentioned objections (19) to the conclusions of Newcomb (17) and Beevers (2), whose interpretations we confirm fully by a different experimental approach. Although a major part of the overall stimulation of O2 uptake elicited by the uncoupler is due to the indirect glycolytic stimulation arising as a consequence of altered intracellular adenine nucleotide concentrations, the rate of respiration in the presence of 10 \( \mu \text{M} \) DNP alone or in the presence of DNP plus pyruvate does exceed the maximum rate achieved with pyruvate alone. Hence, a portion of the DNP effect must be ascribed to a facilitation of mitochondrial oxidation by some means other than merely increasing the intracellular concentration of oxidizable substrate. Such a stimulation of oxidation might be brought about either through removal of a constraint upon electron flow in the respiratory chain or through activation of 1 or more enzymes of the tricarboxylic acid cycle [cf. (11)]. The less marked stimulation elicited by low concentrations of uncoupler (e.g., 5 \( \mu \text{M} \)), can be accounted for wholly in terms of increased substrate for mitochondrial oxidation resulting from the elevated rate of glycolysis.

An additional point to be observed in figures 3a and 3b is that pyruvate, though failing to enhance O2 uptake in the presence of uncoupler, does raise the rate of CO2 evolution appreciably. This fact argues against the possible alternative interpretation that the failure of pyruvate to stimulate oxygen consumption in the presence of DNP might result from a DNP-inhibition of pyruvate uptake by the cells. Such inhibition has, in fact, been reported to occur in the presence of DNP concentrations supraoptimal with respect to O2 uptake (12). However, this hypothesis would not be adequate to explain the data presented here. If DNP were to prevent the entrance of pyruvate into the cells, one would expect that the latter compound would exert no physiological effect whatsoever in the presence of the uncoupler. In the present case, however, the observed stimulation of CO2 evolution by pyruvate in the presence of the uncoupler suggests that the pyruvate does enter the cells and there undergoes nonoxidative decarboxylation. There is consequently an increase in R. Q. from a value of 1.05 in the presence of uncoupler alone to a value of 1.20 when pyruvate is also present, the rise in R. Q. occurring without any increase in the rate of O2 uptake (fig 3b). Additional experiments have shown that 10 mM pyruvate alone leads to an elevated R. Q. with respect to controls in buffer.
only, though in this case there is also a stimulation of \( O_2 \) uptake corresponding to that in figure 2. The chief point to be emphasized as regards the R. Q. data is that they indicate that pyruvate does enter the cells in the presence of DNP as well as in its absence.

The failure of DNP alone to increase the R. Q., though stimulating glycolytic pyruvate production to the point where an excess is present with respect to mitochondrial oxidation, might be understandable if in \( \textit{Acer} \) cells as in yeast (9) the \( K_m \) for the pyruvate dehydrogenase system were much lower than the \( K_m \) of the nonoxidative decarboxylase enzyme. Under these circumstances, the intracellular pyruvate concentration achieved in the presence of 10 \( \mu M \) DNP would presumably be sufficient to saturate only the oxidative enzyme complex. It is also possible that an initial accumulation of acetate inside the presence of DNP, such as that reported by Beevers (2), might tend to inhibit pyruvate decarboxylase activity [cf. (11)]. Additional data would be needed to clarify this question.

The above conclusions, which were initially made purely on the basis of manometric measurements, have received support from direct measurement of intracellular pyruvate concentrations. Figure 4 summarizes data from experiments in which cells were treated with 2 concentrations of DNP under aerobic conditions or subjected to anoxia by flushing the Warburg flasks with high purity \( N_2 \) and keeping them sealed for an additional hour prior to deproteinizing the cell suspension. It is clear that dinitrophenol treatment does indeed result in significantly higher pyruvate concentrations within the cells. The increase brought about by 10 \( \mu M \) is roughly 2-fold, while that brought about by 5 \( \mu M \) is less marked. These elevated pyruvate levels are, however, both somewhat lower than that which prevails under anoxic conditions. The latter exceeds the aerobic control value by several fold. A DNP-induced increase in pyruvate level has also been found by Bianchetti and Marre (4) in pea tissue.

In conclusion, the data taken as a whole suggest that the respiration rate of intact cultured cells of \( \textit{Acer pseudoplatanus} \) is limited by the quantity of substrate (pyruvate) supplied to the mitochondria through glycolysis. Glycolysis in turn is regulated by the intracellular levels of either the adenine nucleotides or \( P_i \). When oxidative phosphorylation is inhibited by dinitrophenol, the glycolytic flux is accelerated to the point where it no longer limits the rate of \( O_2 \) uptake.

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Literature Cited


