

Effect of Adenine Nucleotides on Levels of Glycolytic Intermediates during the Development of Induced Respiration in Carrot Root Slices

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ABSTRACT

Incubation of freshly cut carrot tissue in Na_2ADP and Na_3ATP promotes a marked intracellular increase in both ADP and ATP. The rapid increase in ATP during an ADP incubation and in ADP during an ATP incubation results from the activity of cytoplasmic enzyme systems upon the nucleotide absorbed into the cell from the incubation medium. There is a crossover at the pyruvate kinase reaction, but not at phosphofructokinase, when either ADP or ATP is added to freshly cut tissue. In tissue slices washed in distilled water, pyruvate kinase exhibits a negative crossover in the first 2 hours and a positive crossover between 2 and 10 hours after cutting. Cutting induces large changes in levels of nucleotides and glycolytic intermediates. There is an immediate depletion of these compounds upon cutting, so that nucleotides are added to a system where respiration rate is limited by endogenous nucleotide level.

Variation in respiratory values for fresh cut tissue can be explained in terms of a range of endogenous ADP levels in different tissue batches. Nucleotide incubation experiments are discussed in relation to the provision of ADP to rate-limiting pyruvate kinase during the first phase in development of the induced respiration.

The marked stimulation of oxygen uptake in freshly sliced carrot root phloem parenchyma by sodium salts of ADP and ATP follows an apparent rapid uptake into the metabolic phase of the cells (1). As the effect cannot be explained by the active uptake of the sodium ions, Adams (1) suggested that respiration was stimulated by an increased endogenous concentration of ADP at the site of an ADP-limited enzyme.

In an earlier report, Adams and Rowan (2) described a sequential stimulation of pyruvate kinase and phosphofructokinase in the development of induced respiration following cutting. Endogenous changes in glycolytic intermediates in the 1st day after cutting were consistent with rate limitation of pyruvate kinase by a depletion of adenine nucleotides. In addition to enhanced respiratory rate, slicing of carrot root initiates a developmental sequence of increased polyribosome (8), RNA (8), enzyme (12, 14), and mitochondrial (9, 13) and total protein synthesis (8, 11). These synthetic activities lower cytoplasmic levels of adenine nu-

cleotides, and increased mitochondrial phosphorylation would further limit the supply of ADP at the site of glycolytic enzymes.

In order to investigate metabolic activity of applied nucleotides, intracellular concentrations of adenine nucleotides, inorganic phosphate, and glycolytic intermediates were estimated after incubation of tissue in solutions of sodium salts of ADP and ATP. The nucleotides were taken up by carrot tissue, and changes in EMP² intermediates indicated that in the cytoplasm they relieve a rate limitation at the pyruvate kinase reaction.

MATERIALS AND METHODS

Neutralized perchloric acid extracts were prepared from carrot root phloem parenchyma, and respiration rates were measured as described previously (2). Mature roots of the cultivar Yates All Seasons grown from a common seed source were used in all experiments. To determine changes initiated by slicing, phosphate intermediates were extracted from 20-g tissue blocks cut from whole roots. Comparable extracts were prepared from phloem parenchyma rings (1 mm thick) cut from the same region of the root and washed for 0.25, 0.5, 1, 2, 4, 8, 16, 24, 36, and 48 hr in distilled water.

Twenty-gram samples of freshly cut tissue slices washed for 1 hr were incubated in 2.5 mM Na_2ADP , 2.5 mM Na_3ATP , and water for 0, 1, 2, 4, 5, and 10 min before draining, freezing in liquid nitrogen, and completing the extraction procedure. ADP and ATP at this concentration promoted optimal stimulation of oxygen uptake in freshly cut slices (1).

Phosphate esters, pyruvate, orthophosphate, ATP, and ADP were estimated by the enzymic and firefly assay systems described previously (2). Enzymes and reagents were supplied by Sigma Chemical Company and Boehringer and Sons.

RESULTS

Rapid changes in key metabolites of the EMP pathway occur when mature carrot roots are sliced and washing is commenced in aerated distilled water (Fig. 1).

Changes in adenine nucleotides during ATP and ADP incubations are compared with changes during water incubation in Figures 2 and 3. Effects of incubation in 2.5 mM Na_2ADP on P_i and glycolytic intermediates are presented in Figure 2 and effects of incubation in 2.5 mM Na_3ATP in Figure 3.

DISCUSSION

There is a 40-fold increase in intracellular ADP following incubation of tissue slices in 2.5 mM Na_2ADP . The time course of

² Abbreviations: EMP: Embden-Meyerhof-Parnas; PK: pyruvate kinase; PFK: phosphofructokinase; G6P: glucose-6-P; F6P fructose-6-P; PEP: phosphoenolpyruvate; PYR: pyruvate; FDP: fructose-1,6-P; TP: dihydroxyacetone-P + glyceraldehyde-P; DHAP: dihydroxyacetone-P; GAP: glyceraldehyde-P; 6PG: 6-P-gluconate.

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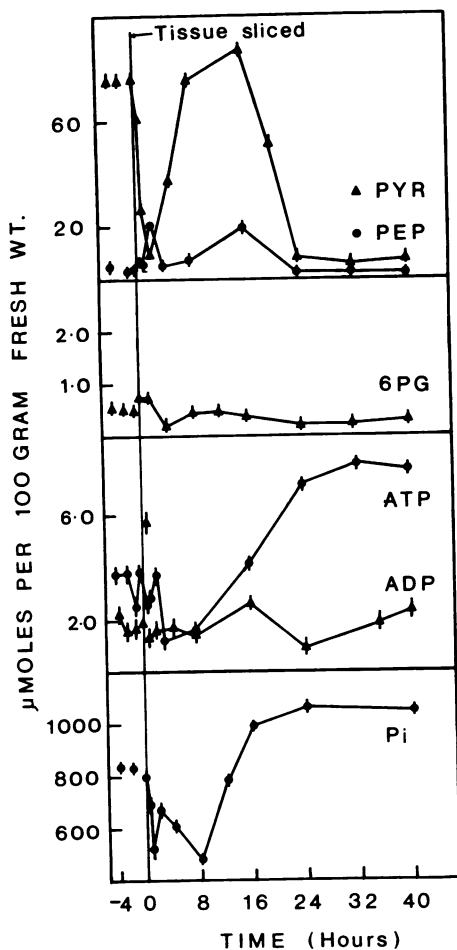


FIG. 1. Effect of slicing carrot root and washing of slices on endogenous levels of pyruvate, PEP, 6PG, ATP, ADP, and P_i .

this increase corresponds with a 4- to 5-min transition phase in respiration rate to a steady elevated value (1). As there is also a significant change in concentration of ATP after 2 min of ADP incubation (Fig. 2), the ADP increase illustrated in Figure 2 represents a cytoplasmic uptake as well as entry into free space. An increase in ADP measured in the tissue is expected because of incomplete removal of incubation solution by draining before extraction. However, in an ADP incubation experiment, the continued increase in ADP over the same course as the ATP increase indicates intracellular uptake; and the ATP increase itself cannot result from contamination by incubation medium. The latter observation would be expected, if part of the ADP is phosphorylated immediately after passing through the cell membrane.

Uptake of large molecules into the cytoplasm of roots has been confirmed by measuring changes in metabolites involved in protein synthesis and respiration (5). Nucleotide uptake into carrot tissue results in marked changes in the substrates and products of rate-regulating glycolytic enzymes. Figure 2 illustrates a crossover at PK in the 1st min of ADP incubation, followed by massive synthesis of pyruvate and an increase in PEP. The freshly cut slices used in ADP incubation contained very low levels of ADP (Table I) which may limit the rate of the EMP pathway. The increase in pyruvate and ATP during ADP incubation indicates that a large portion of the ADP entering the cell is initially phosphorylated by cytoplasmic enzymes, particularly pyruvate kinase. This is a possible explanation for the observed ATP increase during ADP incubation. Stimulation of pyruvate kinase, the rate-limiting enzyme in freshly sliced carrot tissue, would also account

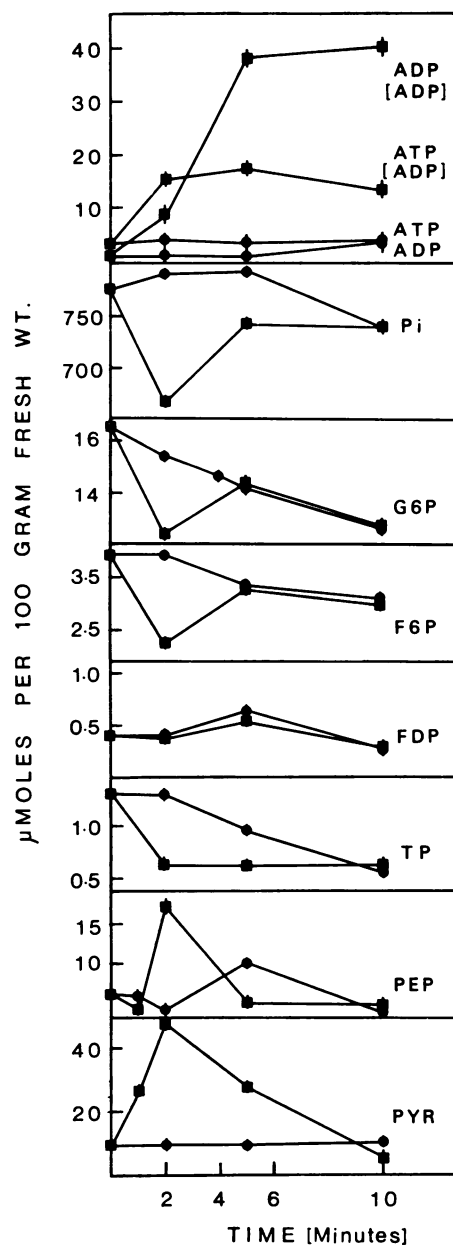


FIG. 2. Effect of incubation of freshly cut carrot slices in 2.5 mM Na_2ADP for 10 min upon levels of ADP, ATP, P_i , and EMP intermediates. Slices washed in distilled water for 1 hr before incubation. ■ Tissue incubated in 2.5 mM ADP; ●: controls incubated in distilled water. ADP (ADP); ATP (ADP): Changes in ADP and ATP during ADP incubation.

for increased O_2 uptake when freshly cut slices are treated with ADP. (If the majority of exogenously applied ADP entered the mitochondria, an increased oxidation of pyruvate would lower the level of pyruvate.) Increases of pyruvate and PEP during ADP incubation indicate that phosphorylation by mitochondria is less important than that of the cytoplasm. A secondary increase in mitochondrial oxidation could return pyruvate and PEP to control values after 10 min of ADP incubation.

Initial declines and recovery to control levels in DHAP + GAP (TP), G6P, and F6P during ADP incubation are in accord with an over-all increase in the rate of the EMP pathway. There is no evidence from F6P and FDP levels for a cross over at PFK at any time during ADP incubation. A part of the large fall in

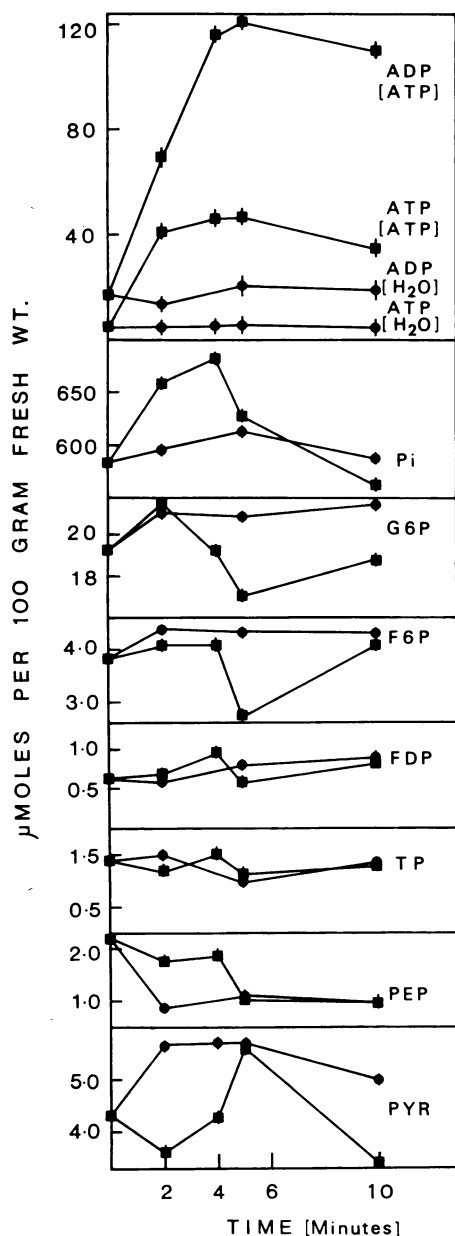


FIG. 3. Effect of incubation of freshly cut carrot slices in 2.5 mM Na_3ATP for 10 min upon levels of ADP, ATP, P_i , and EMP intermediates. Slices washed in distilled water for 1 hr before incubation. ■ Tissue incubated in 2.5 mM ATP; ●: controls incubated in distilled water. ATP (ATP); ADP (ATP): Changes in ATP and ADP during ATP incubation.

P_i (95 $\mu\text{moles}/100$ g fresh wt) is expected to accompany the phosphorylation of the influx of ADP. Organic phosphate reserves in the nonmetabolic compartment of the cell could replace newly esterified phosphate, as has been observed in *Sproidela* (4).

Incubation of freshly cut tissue in 2.5 mM Na_3ATP raised the cellular ATP concentration by 50 $\mu\text{mole}/100$ g fresh weight (Fig. 3), approximately the same amount as determined for the ADP increase in 2.5 mM Na_3ADP incubation medium. In addition, there was a remarkable increase in 100 $\mu\text{moles}/100$ g fresh weight in ADP, so that total nucleotide uptake was greater from 2.5 mM Na_3ATP incubation. A rapid respiratory transition phase and changes in EMP intermediates, as in the ADP incubation, indicate that ATP is also rapidly incorporated into the cell. From the

parallel increases of 95 and 100 $\mu\text{moles}/100$ g fresh weight for P_i and ADP, respectively, it is concluded that much of the exogenously applied ATP is converted directly to ADP by phosphatase activity, either in the cytoplasm or in free space.

Soluble and cell wall bound adenosine triphosphatases, indistinguishable from acid phosphatases, have been isolated from freshly cut carrot root (3, 7). Ninety-five per cent of the hydrolysis product formed from 2 mM Na_2ATP by adenosine triphosphatase activity is ADP; and 2 mM Na_2ADP as substrate is hydrolyzed at 38 per cent of the rate for 2 mM Na_2ATP (3). Incubation of tissue in Na_3ATP or Na_3ADP provides both substrate and ions for salt-stimulated adenosine triphosphatase located at or near the plasmalemma. Greater nucleotide uptake from an ATP compared with an ADP incubation medium and marked increase in endogenous ADP concentration (Fig. 3) can be attributed to hydrolysis of the more active substrate (ATP) by salt-stimulated adenosine triphosphatase.

The time course of F6P changes and constant FDP levels (Fig. 2) throughout ATP incubation preclude generation of the rapid ADP increase by a stimulation of PFK. These observations are consistent with absence of rate limitation by PFK in freshly cut tissue (2). After about 4 min of ATP incubation, the ADP increase results in a nucleotide status similar to that immediately after commencing ADP incubation, except that total nucleotide is higher in the ATP incubation experiment. Fructose-6-P, G6P, pyruvate, and PEP are constant during the first 4 min in ATP, but subsequently there is a large crossover at pyruvate kinase, and decreases in F6P and G6P, as observed immediately after ADP incubation. Hence, the anomalous stimulation of respiration in freshly cut tissue by ATP results from provision of ADP to the ADP-limited PK reaction. Return of elevated P_i levels to control values may be due to incorporation of P_i into a fraction in another compartment of the cell.

Table I illustrates a correlation between higher respiration rates and higher endogenous ADP (or higher ADP/ATP ratios) in eight batches of freshly sliced roots. Large variations in ADP/

Table I. Respiration Rate and Adenine Nucleotide Concentration in Freshly Sliced Tissue Prepared from Eight Separate Batches of the Cultivar Yates All Seasons

Extracted and respiration rate was recorded after slicing and washing in four changes of distilled water for 15 min.

	Respiration Rate	ATP	ADP	ATP + ADP	ADP/ATP
	$\mu\text{moles O}_2/100$ g fresh wt per min	$\mu\text{moles}/100$ g fresh wt			
1. ¹ Locality A. 1968 season	8.1 ± 0.05	4.8	17.2	22.0	3.6
2. Locality A. 1969 season	9.4 ± 0.05	3.5	10.9	14.4	3.1
3. Locality A. 1969 season	7.4 ± 0.05	3.0	8.3	11.3	2.8
4. Locality A. 1969 season	6.2 ± 0.05	2.9	8.2	11.1	2.8
5. Unknown variety (1969)	5.7 ± 0.05	2.6	7.0	9.6	2.7
6. ² Locality A. 1968 season	5.0 ± 0.05	3.6	1.4	5.0	0.4
7. Locality A. 1968 season	4.2 ± 0.05	2.7	1.3	4.0	0.5
8. Locality B. 1967 season	4.0 ± 0.05	4.3	1.3	5.3	0.3

¹ Experimental material used in ATP incubation.

² Experimental material used in ADP incubation.

ATP ratio are indicated in freshly cut carrot tissue, even within one tissue batch or two similar batches of the same cultivar grown during the same season in a common locality. Ratio of ADP to ATP can vary according to the effectiveness of the extraction technique in preventing phosphatase activity (4), but in all experiments reported here, the same extraction procedure was employed. Variation in endogenous ADP content accounts for the range of respiration rates (4–10 μ moles of O_2 per 100 g fresh wt per min.) recorded in different mature roots of a single cultivar, and for reports of "high" and "low" rate respiration responses to cutting. Rowan (10) measured low and high O_2 pressures in the internal atmosphere of roots with high and low respiration rates. Dalgarno and Birt (6) could not isolate tightly coupled mitochondria (high P:O ratios and QO_2 values for succinate and malate) from carrots harvested in July and August. They were first to demonstrate that binding of free fatty acids by serum albumin enables isolation of plant mitochondria with respiratory control. The higher ADP levels and respiratory rates reported in Table I would result if mitochondria were uncoupled by high levels of the free fatty acids liberated upon slicing (6).

Adding 2.5 mM Na_3ADP increased freshly cut tissue respiration from 5.0 to 8.3 μ moles of O_2 per 100 g fresh weight per min, and ADP concentration in this tissue was very low at time of sampling (Table I). In the ATP incubation, ADP concentration in the root selected was an order of magnitude higher (Table I). This difference accounts for the higher initial respiration rate in the ATP experiment, and smaller respiratory stimulation (26%) compared with the ADP incubation (66%). Hence, ATP incubation provides an excess of ADP, much of which is ineffective in stimulating respiration because secondary inhibitory effects may be induced, or other factors may become rate-limiting.

Experiments with intact tissue blocks indicate that over a period of hours, levels of PEP, pyruvate, 6PG, F6P, G6P, FDP, and P_i in intact tissue are relatively constant (Fig. 1). Immediately upon cutting, pyruvate, ATP, and P_i decrease, and subsequently there are large changes in all intermediates except 6PG. The large fall in pyruvate and rapid fluctuations in ADP in the 1st hr after cutting implicate a stimulation by slicing of mitochondrial oxidation. A negative crossover at PK is associated with the initial respiratory decrease before steady respiratory increase commences at about 2 hr after cutting (1). Although cutting initiates the continuous changes previously observed in EMP intermediates (2), it apparently does not initiate a significant pentose phosphate pathway activity (Fig. 1).

Decrease in ATP and continued low levels of ADP and ATP during the first 8 hr after cutting (Fig. 1) confirm that adenine

nucleotides were added to actively synthesizing slices with endogenous limitations on respiration rate. After 24 hr, ATP levels are restored, but ADP does not rise to high values until the tissue slices are 48 hr old (2). The positive PK crossover of day 1 (2) has been further localized to a period between 2 and 10 hr after cutting (Fig. 1). Therefore, added nucleotides induced a crossover at the beginning of the period when crossover at this enzyme is normally observed in untreated tissue.

The analyses of glycolytic intermediates during washing of carrot slices (2) and after adding adenine nucleotides indicate that increases in turnover at PK and PFK during development of induced respiration may result from increases in adenine nucleotide substrates of these reactions. However, changes in activity of PK and PFK during aging could account for increased turnover. Experiments to determine the influence of effectors on PK activity during development of induced respiration are in progress.

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