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Phosphate Metabolism and Induced Respiration in Washed Carrot Slices[1, 2]

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The increase in respiratory rate of slices cut from storage tissue consists of 2 components. The first occurs rapidly after cutting, while the second, named "induced" respiration by Laties (10, 11), develops slowly for 2 to 4 days. Induced respiration in washed slices of carrot root has frequently been reported. If we assume that sugars only are substrates for the additional respiration after cutting, additional CO_2 must be generated by the Embden-Meyerhof-Parnas (EMP) pathway or the pentose phosphate pathway (2, 3). In this paper, changes in concentration of phosphorylated intermediates and rate of respiration are discussed in relation to regulation of the 2 pathways.

Materials and Methods

From mature carrot roots (*Daucus carota* L.) discs 1 mm × 8 mm were cut using a cork borer and razor blade or sledge microtome and washed in aerated distilled water until extracted or placed in distilled water in the respirometer mounted on glass needles. Respirometer vessels were filter tubes with sinters (width 3.3 cm; height 12 cm) fitted with ground joints and splash heads. CO_2 was removed from the compressed air entering the respirometer by passing through NaOH. The flow of gas (approx. 6 liters per hr) was regulated with a needle valve and flowmeter (quickfit FMO/V). The concentration of CO_2 in the gas stream was automatically recorded from an infrared gas analyser (Sir Howard Grubb Parsons Ltd.).

Acid-soluble phosphates were extracted from discs with cold perchloric acid (15) and the following fractions estimated in the neutralized extracts by the method of Slater (15, 16). A) Hexose monophosphate (HMP): the sum of glucose-6-P, glucose-1-P and fructose-6-P; B) Hexose diphosphate plus triose phosphate (HDP + TP): the sum of fructose-1,6 diP, 3-P-glyceraldehyde and dihydroxyacetone-P; C) High-energy phosphate (~ P): the sum of the γ-phosphate groups of nucleoside triphosphates and the β-phosphate group of ADP. P_i was measured by the method of Berenblum and Chain (6). Total acid-soluble phosphate was measured by the method of Allen (1).

Results

The Immediate Effect of Cutting upon the Concentration of Phosphorylated Compounds. Approximately 1 g of discs were cut from a cylinder of parenchyma, weighed, and phosphorylated compounds extracted as rapidly as possible. Replicate samples were washed and aerated in a large volume of water

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Table I. *Concentration of Phosphate Fractions in Extracts from Unwashed Carrot Discs and from Discs Washed in Distilled Water for 1 Hour.*

Phosphate fraction	Unwashed discs	Discs washed 1 hr	Change after washing
	μmoles per g fr wt		
	Mean S.D.	Mean S.D.	
HDP + TP	0.26 ± 0.01	0.21 ± 0.01	−0.05
HMP	0.50 ± 0.02	0.54 ± 0.02	+0.04
~ P	0.31 ± 0.01	0.17 ± 0.01	−0.14
P_i	2.7 ± 0.14	2.3 ± 0.12	−0.4

for 1 hour, surface-dried, reweighed and extracted. The concentrations of the phosphate fractions determined in duplicate are compared in table I. The concentrations for washed tissue are corrected for cell damage by assuming that as in potato (7) no net conversion of acid-soluble to acid-insoluble phosphate occurred in 1 hour and that all fractions decreased in proportion to the loss of total acid-soluble phosphate during washing. While insignificant changes were seen in the other fractions following this correction the concentration of ~ P decreased significantly from the original value after tissue had been washed for 1 hour.

Changes in Rate of Respiration Immediately after Cutting. Figure 1 shows the change in output of CO_2 on slicing a cylinder of carrot tissue. A cylinder of tissue was excised, rinsed, surface-dried and held at 21° in a humid atmosphere low in CO_2 for 24 hours before being placed in the respirometer. CO_2 output was then continuously recorded from the infrared gas analyser. After 1 hour the tissue was sliced and the slices blotted and placed in the respirometer vessel in 50-ml water. In this experiment CO_2 was evolved steadily from the piece of unsliced tissue at a rate of 1.2 μmole per g fresh weight per hour, less than one-third the minimum rate recorded for the slices in water. Both the apparent fall and the following transitory rise in net rate of CO_2 output following slicing were consistently reproducible in experiments of this kind.

During the first 30 minutes after slicing CO_2 from respiration is not distinguishable from CO_2 released in the course of equilibration with the gas stream in the instrument, thus the rate at which respiration rose initially cannot be determined.

Changes in Concentration of Phosphorylated Compounds during the Development of Induced Respiration. A large batch of discs was washed in 5 liters of aerated water which was replaced at regular intervals. Samples of discs were removed from the batch at 1, 2, 4, 8, 16, 24, 48, 72 and 110 hours and the rate of respiration measured for 1 hour; then phosphorylated compounds were extracted and analyzed. Results of analyses are shown in figure 2;

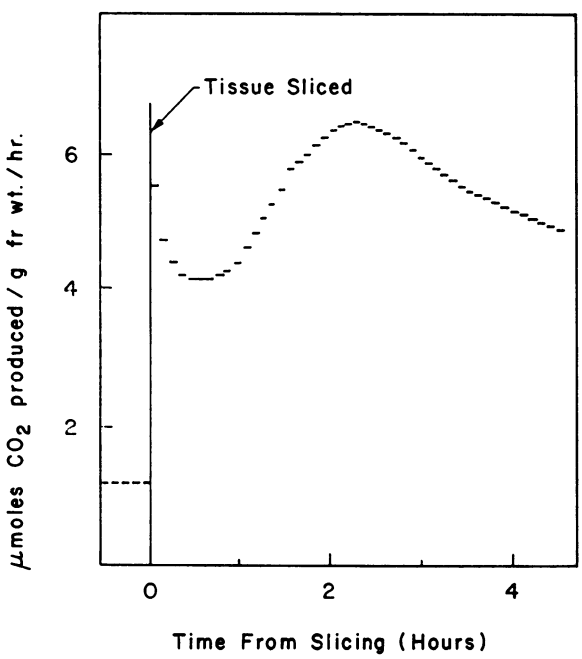

FIG. 1. The rate of CO_2 output by a cylinder of carrot tissue maintained at 21° for 24 hours, then sliced and washed at 21° for a further 4 hours after cutting. Plotted from 6-minute mean rates from chart.

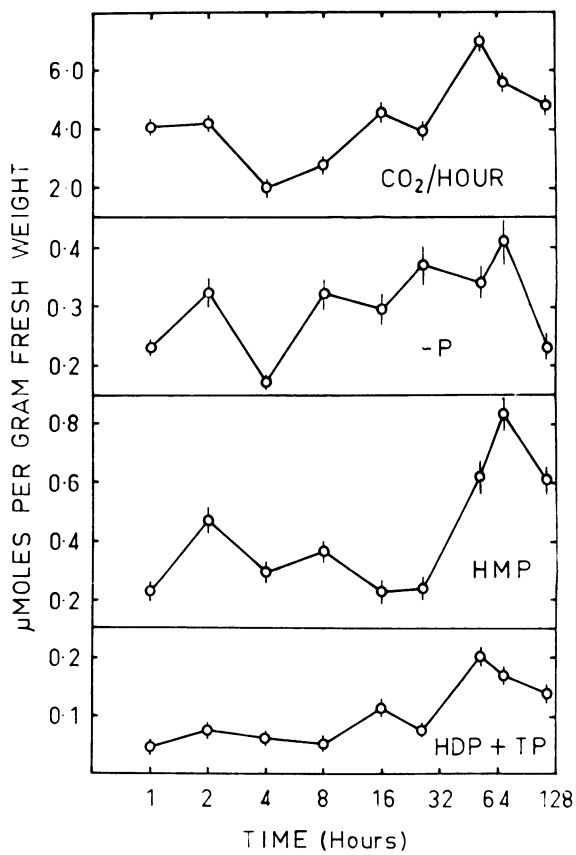

FIG. 2. Respiratory rate and concentration of ~ P, HMP and HDP + TP in discs washed at 21° for 110 hours after cutting. Plotted on a logarithmic time scale.

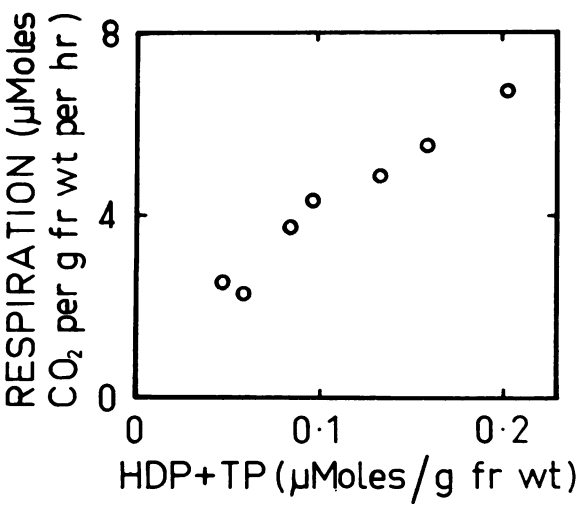

FIG. 3. The correlation between rate of respiration and concentration of HDP + TP in discs washed at 21°. Data of figure 2 from 4 to 110 hours after cutting.

the cumulative error of each determination is shown by a vertical line at each point. In this experiment, the induced respiration developed between 4 and 48 hours after cutting and the rate was correlated with the concentration of HDP + TP (fig 3). ~ P increased throughout this period, while HMP decreased until 24 hours, then increased 4-fold by 48 hours.

Changes in Concentration of Phosphorylated Compounds during the Decline Following Development of Induced Respiration. After the maximum observed at 48 hours, the rate of respiration decreased until the end of the experiment. Again, the concentration of HDP + TP was correlated with rate of respiration.

After the respiratory maximum at 48 hours, HMP continued to increase until 64 hours; then it decreased until the end of the experiment.We observed similar changes in concentration in a second experiment where slices were extracted after washing for 98 and 270 hours.

Discussion

The fractions measured here, HMP and HDP + TP, contain compounds interconverted by reversible reactions in quasi-equilibrium, thus changes in concentration of each fraction should reflect changes in concentration of each compound in the fraction. Changes in glucose-6-P, fructose-6-P, fructose-1,6-diP and dihydroxyacetone-P observed in pea seed (4) support this assumption.

Hess (9) has shown that although the reversible reactions of glycolysis in ascites cells are in quasi-equilibrium, the irreversible kinase reactions are not. When the rate of glycolysis is regulated by concentration of substrate, not enzyme, these irreversible reactions can act as regulators.

Chance, Holmes, Higgins and Connolley (8) have proposed a method, in terms of a crossover theorem, for detecting sites of activation along a sequence of reactions. When turnover is increasing, the concentration of substrate for the regulator reaction decreases while the concentration of the product increases; when turnover is decreasing the opposite changes occur.

Two irreversible dehydrogenase reactions separate the HMP fraction and the HDP + TP fraction of cells possessing the pentose phosphate pathway; these 2 reactions could act as regulators of this pathway. Carrot tissue is known to possess enzymes of both the pentose phosphate pathway and the EMP pathway; however, ap Rees and Beevers (3) have shown that the pentose phosphate produced in carrot slices is metabolized via the EMP pathway and the tricarboxylic acid cycle. The results of the analyses presented here do in fact give evidence for a regulatory step between HMP and HDP + TP but do not distinguish which pathway was activated.

Activation of the EMP Pathway. Regulation of the EMP pathway can be achieved by separation of the pools of cytoplasmic and mitochondrial ATP (4, 9). As long as the affinity of phosphorylating systems for P_i and ADP is higher in mitchondria than in cytoplasm the rate of phosphorylation in the cytoplasm will limit the EMP pathway (5).

A second mechanism known to regulate the EMP pathway is activation or inhibition of phosphofructokinase (13). In crude extracts of parsley and avocado, ADP inhibits this enzyme and P_i reverses the inhibition (12).

Activation of the Pentose Phosphate Pathway. The apparent K_m (glucose-6-P) for glucose-6-P dehydrogenase in extracts of carrot root is 0.7 mM (D. D. Davies, private communication). Although the intracellular concentration of glucose-6-P is not known, the concentration of HMP is lower than 0.7 μmoles per g fresh weight in freshly cut slices and the concentration of glucose-6-P could regulate the activity of glucose-6-P dehydrogenase. Thus, increasing synthesis of glucose-6-P through activation of hexokinase could activate the pentose phosphate pathway.

It is unlikely that an increase in concentration of enzymes of either the EMP or the pentose phosphate pathway accounts for induced respiration, as the respiratory rate of slices treated with 2,4-dinitrophenol is as high with freshly cut as with washed slices (3). As 2,4-dinitrophenol activates the EMP pathway the capacity of the enzymes of this pathway immediately after cutting seems sufficient to carry the induced respiration.

Changes Associated with Induced Respiration. In the experiment shown in figure 2, induced respiration developed between 4 and 48 hours after slicing. As seen in figure 3 the rate of respiration was correlated closely with concentration of HDP + TP during both the development and decline of induced respiration. The regulator reaction therefore comes before HDP + TP in the sequence of reactions. The con-

centration of HMP was tending to fall while HDP + TP fraction was tending to rise during the period of increasing respiration rate up to 16 hours. Opposite changes occurred in these fractions between 48 and 72 hours when respiration rate was falling. If Chance's crossover theorem is applied here, the regulator reaction must have lain between the 2 fractions. Possible activating mechanisms of pathways between these 2 fractions were discussed above. Between 24 and 48 hours, both HMP and HDP + TP increased sharply. Unless the incorporation of carbon into polysaccharide and pectin had decreased, this implies activation of hexokinase.

The Activating Mechanism Giving Rise to the Induced Respiration in Sliced Carrot Root. Ap Rees and Beevers (3) have reported that the fraction of respiration carried by the pentose phosphate pathway increased in washed carrot root slices as induced respiration developed. The changes in concentration of phosphate fractions presented here locate possible sites regulating induced respiration. The changes in phosphate fractions are consistent with activation of both phosphofructokinase (up to 16 hr) and hexokinase (between 24 and 48 hrs). As the activation giving rise to induced respiration does not occur rapidly in contrast to that following inhibition of oxidative phosphorylation in carrot by anaerobiosis (17) and by 2,4-dinitrophenol (14) induced respiration is not caused by rapid inhibition of oxidative phosphorylation upon slicing, notwithstanding the abrupt fall in $\sim$ P concentration after slicing and washing. The slow increase in induced respiration could reflect the complex interaction of ATP, ADP and P_i upon phosphofructokinase (12), or follow an increasing capacity for oxidizing NADPH after slicing, as suggested by ap Rees and Beevers (3).

Summary

The rate of respiration of washed slices from carrot tissue, (*Daucus carota* L.) at 21° was higher than that of unsliced tissue. The concentration of high-energy phosphate decreased markedly within the first hour after slicing.

After 4 hours, the output of carbon dioxide slowly increased until 48 hours, giving rise to the induced respiration. The output of carbon dioxide was correlated closely with concentration of fructose-1, 6-diphosphate plus triose phosphate showing that the regulating reaction preceeded the production of these compounds.

Fluctuating concentration of hexose monophosphate showed that activation of respiration occurred in 2 phases. At first, activation of phosphofructokinase or of the pentose phosphate pathway was indicated. In the second phase, the concentration of hexose monophosphate increased markedly; unless utilization of hexose monophosphate in processes other than respiration decreased, this implies activation of hexokinase.

A reaction in the metabolic pathway prior to production of fructose-1, 6-diphosphate plus triose phosphate appeared to regulate the respiratory rate during its decline after the maximum at 48 hours.

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