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Glycolytic Control of Respiration during Aging of Carrot Root Tissue

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ABSTRACT

Changes in respiration in aging carrot slices are correlated with a characteristic sequence of activation of the irreversible reactions of the glycolytic pathway. The induced "wound" respiration increases in two stages; these appear to correspond to a period of active synthesis, when ADP, produced in the cytoplasm from synthetic reactions, activates pyruvate kinase, and a period of readjustment when ATP, no longer reacting in synthetic reactions, activates phosphofructokinase. Although ATP is cited frequently as a negative effector of phosphofructokinase, unless the concentration of ATP at the site of phosphofructokinase is 50-fold higher than the concentration expressed per unit fresh weight of the tissue, it cannot act in this way in carrot slices. The activation of the oxidative pentose phosphate pathway observed by ap Rees and Beevers is restricted to the first stage of the induced respiration.

When thin tissue slices are cut from underground storage organs of many plants, the immediate and rapid increase in respiration rate is followed by a more gradual rise to a maximum after 2 or 3 days of aging in distilled water. The slower respiration increase, termed "induced" respiration by Laties (20), is a characteristic feature of washed carrot root slices (15, 32).

Induced respiration is only one of a number of metabolic changes initiated by slicing. Invertase activity, absent from mature sugar beet, artichoke, and carrot tissue, is observed in washed slices after a lag period of 6 to 8 hr (7, 14, 34). Fatty acid synthetase (36), RNA (21), polyribosomes (21), phospholipids (31), mitochondria (22, 35), and total protein (30) increase markedly during the first 24 hr of aging. However, since DNP[2] markedly stimulates the respiration of freshly cut carrot slices (4), net synthesis of respiratory enzymes need not underlie development of induced respiration, which is more likely to be due to activation of the EMP pathway or the oxidative pentose phosphate pathway.

In this work we identify regulator reactions of the EMP pathway by applying the crossover theorem of Chance (11). That is, crossover points are located by following changes in the ratio of substrate to product of the irreversible reactions of the EMP pathway. The reactions concerned are hexokinase, PFK, P-glycerate kinase, and PK (18). Analyses of intermediates of the EMP pathway in yeast (16, 25), rat kidney (33, 37), ascites tumor cells (38), and cell cultures of *Acer pseudoplatanus* (17) implicate PFK as a regulator in a variety of experimental treatments. Glycolytic regulation may also be exerted by hexokinase in lens (23), by PK in yeast (19), or by a combination of hexokinase, PFK, and PK in leukocytes (28) and erythrocytes (29). In carrot root slices, there is a characteristic sequence of regulation by glycolytic enzymes during the development and decline of the induced respiration.

MATERIALS AND METHODS

Roots of mature carrots (*Daucus carota* L., cultivar Yates All Seasons) were peeled and cut into slices 1 mm thick, and the xylem parenchyma at the center of each slice was discarded. The rings of phloem parenchyma were aerated at 20 C in frequent changes of distilled water until samples were removed for extraction or for recording rate of respiration. Bacterial contamination was reduced to an insignificant level by changing the water hourly for the first 6 hr after cutting.

Respiration Measurements. A Clark oxygen electrode (Yellow Springs Instrument Co.) was fitted into the side arm of a round respirometer flask connected to a transposition and polarizing circuit, and Hitachi QPD 73 recorder. The instrument was zeroed with pure N_2 gas and the recorder scale adjusted so that water saturated with air at 20 C gave full scale deflection. Slices of parenchyma from carrot phloem were impaled on glass needles mounted in the rubber stopper of the flask, in which constant temperature and homogeneous distribution of oxygen were maintained with a magnetic stirrer. Respiratory traces were periodically recorded at 20 C for 0.5 to 2 hr, and rates of uptake of oxygen were calculated from the initial oxygen content of the flask.

Preparation of Extracts. Sixty grams of tissue frozen in liquid nitrogen were blended in 60 ml of 5% (v/v) $HClO_4$ in an MSE homogenizer for 4 min, and the blend was centrifuged for 30 min at 17,000*g*. The supernatant solution was adjusted to pH 7 with 8 N KOH, and precipitated $KClO_4$ was removed by centrifugation. The extract was then concentrated at low temperature over H_2SO_4 *in vacuo*, and, when necessary, pigments were cleared with charcoal (BDH) at acid pH.

Methods of Analysis. Phosphate esters and pyruvate were estimated in cleared extracts by enzymic analysis (8) and P_i by the method of Allen (3). ATP was determined in the uncleared extract with luciferin-luciferase prepared from firefly tails by procedure c of McElroy (8). With the use of a magnesium-arsenate buffer, light emitted at 562 nm was recorded with an Aminco-Bowman spectrophotofluorometer and Hitachi QPD 73 recorder. ADP was converted to ATP by incubating a 0.02-ml sample of extract with 1.0 μmole of PEP, 25 μmoles of $MgCl_2$, 200 μmoles of KCl, and 0.01 ml of PK (0.25 mg of protein) in a total volume of 0.6 ml.

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[2] Abbreviations: DNP: 2,4-dinitrophenol; 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-diP; TP: dihydroxyacetone-P + glyceraldehyde-P; 6PG: 6-P-gluconate; 2PGA: 2-P-glycerate; 3PGA: 3-P-glycerate; G1P: glucose-1-P.

The sum of ATP and ADP was determined after 30 min of incubation at 20 C, and ADP in the extract was calculated by difference.

RESULTS

Changes in concentration of intermediates and nucleotides during aerobic washing of the tissue slices are shown in Figure 1. Recoveries of authentic substrates added to enzymic assays were within the range of 90 to 100% for G6P, F6P, PEP, PYR, FDP, TP, 6PG, and ADP, and between 85 and 95% for 2PGA and 3PGA. Analysis of two other batches of tissue produced results consistent with the sequence of regulator reactions described below (Fig. 4). In particular, the crossovers at PK (Fig. 1d) at day 1 and PFK on day 3 (Fig. 1c) occurred in other experiments. Changes in ADP and ATP were also reproducible.

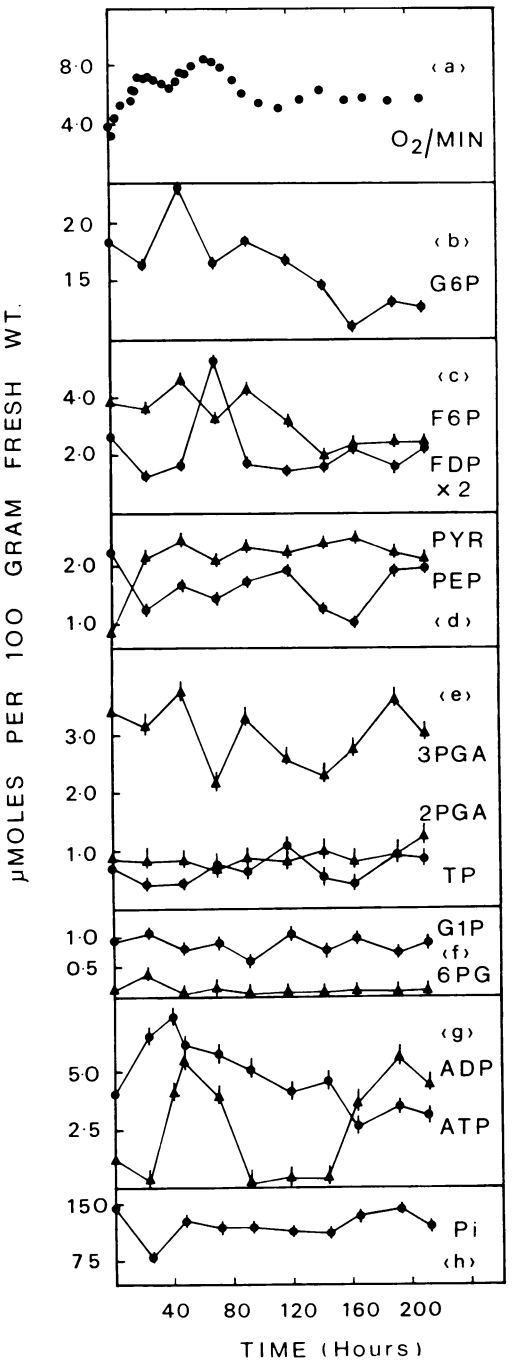

FIG. 1. Respiratory rate and changes in concentration of glycolytic intermediates, 6PG, adenosine pyrophosphates, and P_i in slices of carrot tissue washed in distilled water for up to 220 hr after cutting. Vertical bars represent the range of values in assays of each extract.

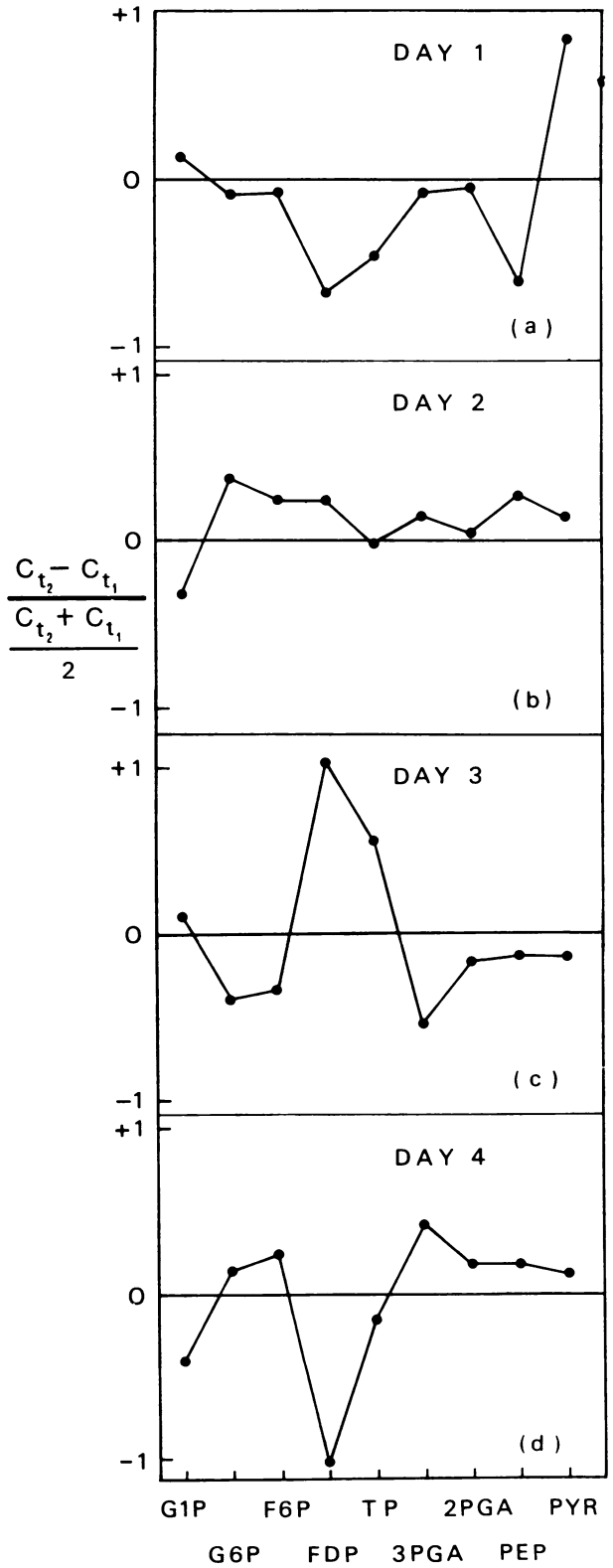

FIG. 2. Crossover diagrams for glycolytic intermediates during the first 4 days after cutting, where C_{t_1} and C_{t_2} are concentrations of the metabolites on consecutive days.

DISCUSSION

The crossover theorem (11) can be used to correlate changes in carbon flux in washed carrot slices with stimulation or inhibition of the irreversible reactions (18) regulating the rate of respiration. Crossover diagrams (Fig. 2) and phase plane diagrams (Fig. 3) prepared from the data in Figure 1 assist in detecting where and when crossover occurs. In the phase plane diagrams, where the simultaneous concentration of two intermediates measured at different times from cutting are plotted against each other, the angle of incidence of the line joining the points is 45° when the changes in concentration are in phase and 135° when the changes

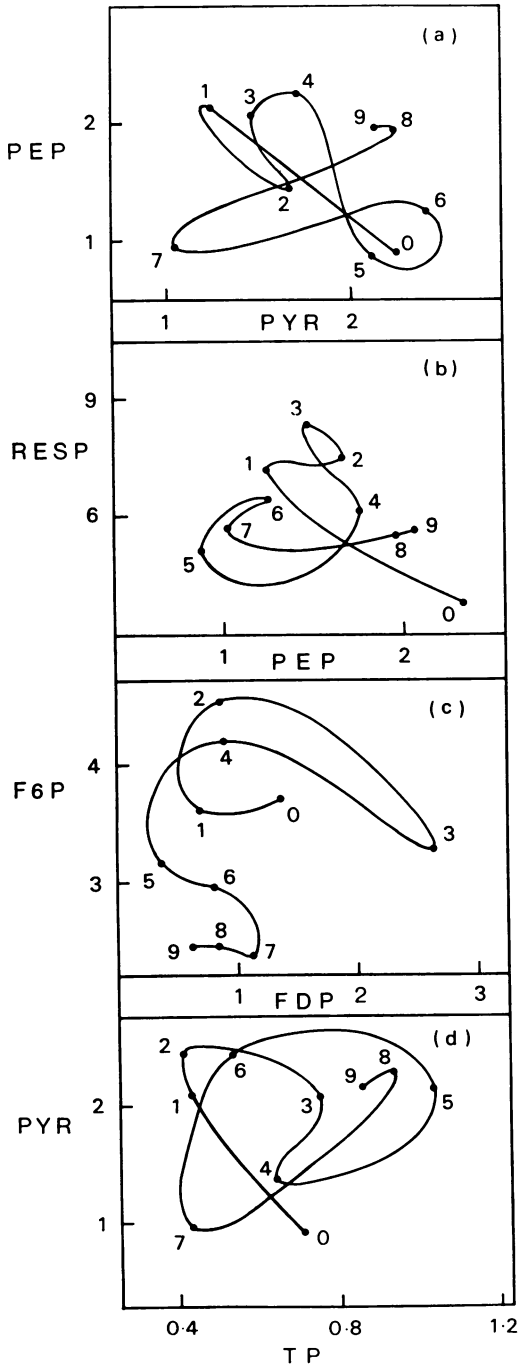

FIG. 3. Phase plane plots of respiratory parameters measured for 9 consecutive days in slices washed in distilled water. All units are micromoles/100 g fresh weight.

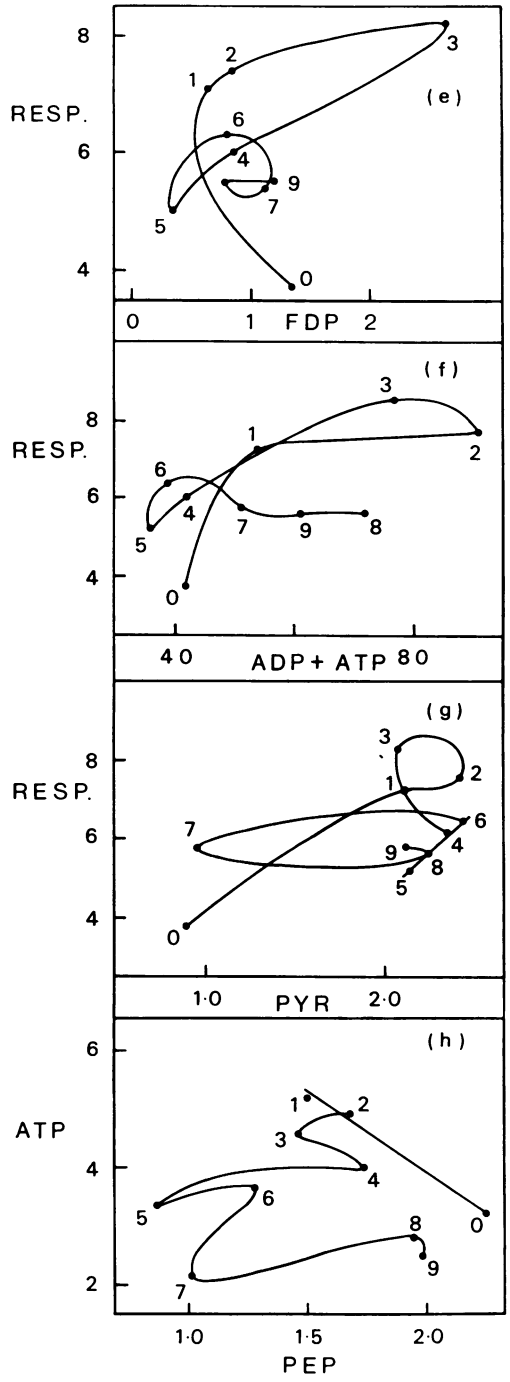

FIG. 3 e-h

are out of phase (*i.e.*, when a crossover occurs). Most of the figures are ellipsoids or spirals, indicating that the metabolites concerned do not rise and fall simultaneously. In these figures the angle formed by the longer axis of the diagram represents the dominant phase relation (9). A phase change in a diagram results when there is a change in the control point of the EMP pathway during washing.

Both ATP and ADP occur in at least two phases of the cell (cytoplasm and mitochondria) and are separated by diffusion barriers; thus, changes in *total* concentration of adenosine pyrophosphates cannot be associated with crossover phenomena. Changes in concentration of adenosine pyrophosphates in the

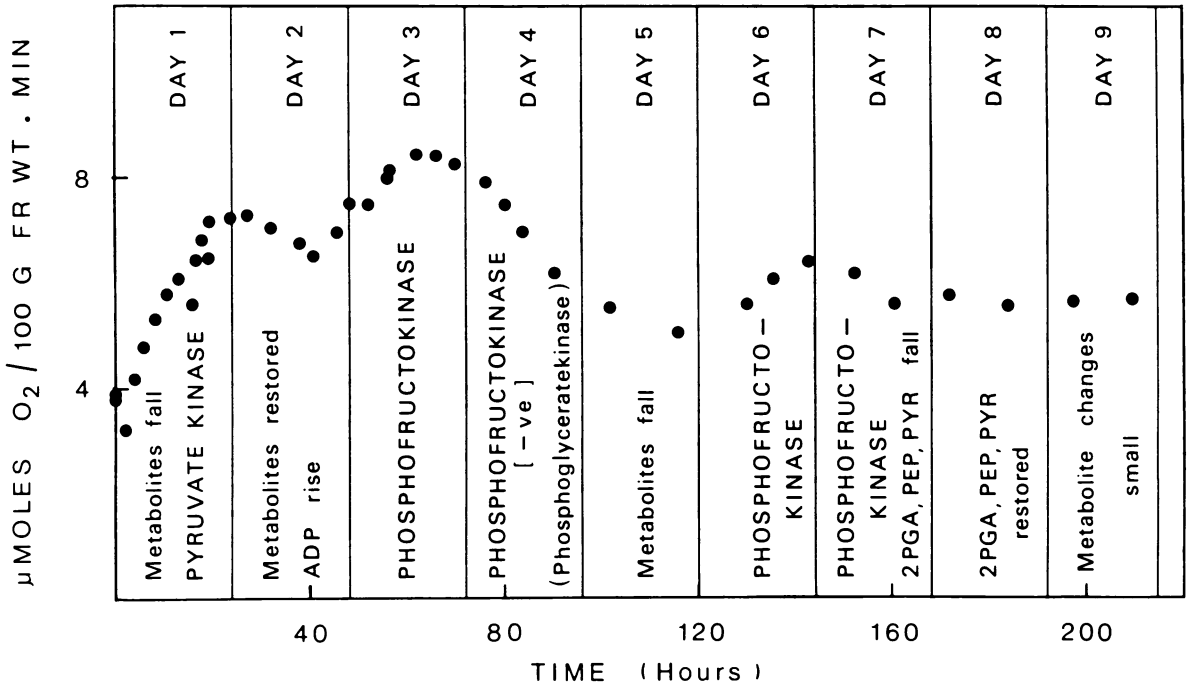

FIG. 4. Sequence of major regulator reactions (indicated in upper case) and changes in metabolites in slices washed for 220 hr in distilled water.

cytoplasmic phase must be deduced from the crossovers as they occur.

Induced Respiration. In the experiments reported here, the rate of the induced respiration increases in two stages, showing separate peaks at 24 and 60 hr (Figs. 1, 4). These peaks, occurring in all other experiments, are associated with control at different regulator reactions, since crossovers occur at PK during day 1 (stage 1), and at PFK during day 3 (stage 2) (Figs. 2, 3). The out-of-phase relationship of PYR and PEP (Fig. 3a), PEP and ATP (Fig. 3h) and of PEP and respiration (Fig. 3b), and the in-phase relationship of PYR and respiration (Fig. 3g) confirm that PK regulates respiration during stage 1. A negative crossover between F6P and FDP at day 4 (Fig. 2, 3c) confirms that PFK is the main regulator reaction during stage 2. Changes in phase relation occur when PFK replaces PK as regulator in the second stage (Figs. 3a, 3c, 3d, 3e, 3g). The in-phase relation between respiration and FDP (Fig. 3e) and out-of-phase relation between F6P and FDP (Fig. 3c) are consistent with PFK control.

We postulate that the respiratory increase during stage 1 is a result of cytoplasmic energy-requiring processes initiated by slicing. As already mentioned, several synthetic processes are reported in slices during the first 24 hr after cutting (7, 14, 21, 30, 34–36), and Click and Hackett (12) concluded that the induced respiration was a consequence of the concomitant protein synthesis in potato slices. In particular, in sliced carrot root, the proportion of ribosomes present as polyribosomes increases from 10 to 30% 1 hr after cutting, and to 60% after 6 hr (21). We suggest that increasing concentration of ADP produced in synthetic processes in the cytoplasm induces the crossover at PK. However, net synthesis of *total* adenosine pyrophosphate occurs during stage 1 (Fig. 1g), and we conclude that the synthesis of ATP from ADP in the mitochondria obscures the increase in concentration of ADP in the cytoplasm. Adams (1, 2) provides direct evidence that adenosine pyrophosphate limits rate of cytoplasmic phosphorylation and respiration in freshly cut carrot slices. Stimulation of O_2 uptake by addition of pyruvate, the product of PK, to freshly sliced tissue (1) confirms the role of this enzyme in the respiratory increase during the 1st day of washing.

The onset of the second stage of the induced respiration at 40 hr coincides with a sharp fall in the concentration of total adenosine pyrophosphate. We conclude that an increase in cytoplasmic ATP, no longer used in synthetic reactions or provided by nucleotide synthesis, activates PFK. The increase in total ADP during day 3 apparently is a consequence of the decrease in rate of synthesis of ATP in the mitochondria.

The increase in respiration rate at 140 hr (Figs. 1, 4), although small, is reproducible and is associated with a crossover at PFK (Fig. 3c). This peak cannot be related to any known process in the cell, but it precedes a period of net synthesis of total ADP plus ATP (Fig. 1g). After this peak, the slices respire at a steady rate until their breakdown, although intermediates continue to oscillate without control point characteristics.

Many workers have examined allosteric modulation of regulator reactions (5, 24), and we cannot neglect this mechanism in explaining stimulation and inhibition of regulator reactions in carrot tissue. At concentrations above 1 mM and with Mg^{2+} at 4 mM, ATP, ADP, AMP, and citrate are negative effectors and P_i is a positive effector of PFK in carrot (13). Displacement of the PFK reaction from equilibrium and accumulation of FDP in anoxia have frequently been ascribed to ATP inhibition of this reaction (17, 27). In the present experiment, the concentrations of ATP or ADP expressed per unit fresh weight do not exceed 72.5 μM, and they could act as negative effectors only if the cytoplasmic phase of the cell to which they were restricted was as little as 2% of total cell volume. If ATP in the cytoplasm was sufficiently concentrated, a change in sensitivity to ATP acting as a negative effector as observed in artichoke tuber slices (10) could account for the stimulation of PFK. The concentrations of ATP reported in this experiment are comparable with those reported elsewhere (6). The concentration of citrate in the cytoplasmic pool in carrot has been reported as 50 μM (26); this concentration also appears low for citrate to act as negative effector. Concentration of P_i does not change during day 3 and is unlikely to act as a modulator of PFK at this time.

Throughtout most of the experiment the concentration of 6PG was low (Fig. 1), but it increased at 24 hr when G6P decreased

slightly, thus constituting a crossover at G6P dehydrogenase; this did not persist beyond stage 1 and is consistent with the stimulation of the oxidative pentose phosphate pathway reported in carrot slices during the first 10 hr of washing by ap Rees and Beevers (4).

Figure 4 summarizes the conclusions we draw from Figures 1, 2, and 3 about the sequence in which reactions (PK and PFK) regulate respiration in sliced carrot root tissue washed in water. Experiments testing the proposed mechanisms of activation of these regulator reactions will be presented in subsequent papers (1, 2).

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