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Metabolic Regulation in the Senescing Tobacco Leaf

II. CHANGES IN GLYCOLYTIC METABOLITE LEVELS IN THE DETACHED LEAF

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P. K. MACNICOL
Division of Plant Industry, Commonwealth Scientific and Industrial Research Organization, Canberra, A.C.T. 2601, Australia

ABSTRACT

Yellowing of detached mature tobacco leaves standing in water in the dark was accompanied by a strong "climacteric rise" in respiration rate. During this period the ATP level and energy charge of the adenylate system also rose. The levels of glycolytic intermediates between glucose 1-phosphate and triose phosphates rose, those between 3-phosphoglycerate and phosphoenolpyruvate fell, and pyruvate rose. On the assumption of a drop in NAD/NADH ratio, as found by other workers in wheat leaves, the reverse crossover between triose phosphates and 3-phospholglycerate was attributed to inhibition of glyceraldehyde 3-phosphate dehydrogenase. The forward crossover between phosphoenolpyruvate and pyruvate was taken to indicate activation of pyruvate kinase, possibly by fructose diphosphate. Secondary large rises in pyruvate and fructose diphosphate occurred well after the climacteric peak had been passed. No evidence was found for participation of phosphofructokinase in metabolic control in the yellowing leaf. Possible limitations to the use of the crossover theorem in the present situation, such as changes in compartmentation and in flux through branch points, are emphasized.

Blackman's pioneering studies (13) established that the drift in dark respiration has the same basic form in detached mature leaves of both woody and herbaceous plants. This consists of an initial fall to a transitory minimum followed by a strong rise as yellowing sets in (the "senescent phase"), succeeded by a fall as cell organization collapses. In some species, attack by saprophytic microorganisms produces a secondary rise in respiration rate. In this paper the expression "senescent phase" will be replaced by the more appropriate "climacteric rise," a term borrowed from the literature of fruit respiration (12). The presence and extent of the climacteric rise depend critically on the physiological age of the leaf (9, 13), older and younger leaves often showing no rise at all. The striking changes in chemical composition of such starving leaves include rapid breakdown of chlorophyll, starch, protein, RNA, and DNA, with increases in levels of sugars, amino acids, and amides (13, 34). Notwithstanding the catabolic trend of these changes, the specific activities of some enzymes increase markedly (5, 6, 31).

In the first stage of flue-curing of tobacco, known as "coloring," the detached ripe leaves are allowed to yellow in a humid atmosphere for several days in the dark at 35 to 40 C. During this period compositional changes take place which are essential for the desired smoking characteristics of the product (10). These changes, which include rapid starch and protein hydrolysis (3), a transitory rise in respiration rate (2), and temporary increases in the activity of polyphenol oxidase and peroxidase (33), are qualitatively so similar to those of the nonwilting starving leaf (14, 32) that the latter can be used as a model system for this stage of curing (10, 33).

In the previous paper (17) evidence was given that discs from yellow, detached tobacco leaves, unlike discs from mature green leaves, incorporate $^{32}P_i$ into respiratory metabolites by an enhanced aerobic glycolysis. Moreover, differences in the quantitative pattern of labeling of the metabolites suggested that their relative endogenous levels changed during yellowing. These findings prompted a critical examination of the levels of glycolytic intermediates during the climacteric rise of the detached leaf, with the aim of defining control points in the overall process by use of the crossover method developed by the Chance school (8, 11). Here the percentage changes in levels of intermediates relative to their mean values are plotted in the linear sequence in which the enzymic reactions occur. A "forward crossover" (depletion of substrate with accumulation of product) during increasing flux through the pathway indicates a control site which is activated; conversely a "reverse crossover" with decreasing flux indicates an inhibited control site. Application of this approach to the metabolite data for yellowing detached leaves has enabled tentative identification of one site of changed control and one of flux limitation.

MATERIALS AND METHODS

Plant Material and Treatment of Excised Leaves. Tobacco plants (*Nicotiana tabacum* L., cv. Virginia Gold) were grown in the C II mixture of Matkin and Chandler (19) (a sand-peat moss-fertilizer medium) in a glasshouse controlled to 29 to 32 C (day) and 21 to 24 C (night). From the time of transplanting into 2-gallon buckets until the flowers opened, supplementary nutrient was given in the form of two tablets per week (Gard-N-Tabs from Amalgamated Chemical Industries, Bankstown, N.S.W.). Leaves of area 1000 to 1200 cm^2 at positions 8 to 10 on the stem (counting the first leaf above the cotyledons as 1) were excised and stood in distilled water in a 25 C darkroom so that the midrib was supported.

Measurement of Respiration Rate. The leaf was enclosed in a chamber of 3.04-liter volume incorporating a small water reservoir for the petiole. The chamber was placed in a 25 C dark growth cabinet. Humidified air was passed over the leaf at a flow rate of 3 to 4 liters/min, and the differential in CO_2 concentration between this air stream and one passing through a dummy chamber of the same volume was measured with a

Grubb Parsons infrared gas analyzer with lead telluride filters, coupled to a recorder. The respiration rate was measured over a 1-hr period and corrected for loss of area as discs. The system was calibrated with a Wösthoff gas-mixing pump.

Estimation of Chlorophyll Content. A nondestructive photometric method was used (unpublished), in which absorption of red light by a 1-cm² area of leaf is measured using a cadmium sulphide cell. Twelve readings were taken on each leaf and averaged, then converted to total chlorophyll content with the aid of a calibration curve relating instrument readout to chlorophyll content of 80% acetone extracts, previously obtained with the same tobacco cultivar.

Leaf Sampling and Analysis of Metabolites. Each day following measurement of respiration rate and chlorophyll, the leaf was laid upper surface downwards on a flat surface, and a set of 15 1-cm² discs was removed with a sharp punch according to a scheme (unpublished) based on random permutations of 10. Major leaf veins were carefully avoided. Each disc was was dropped immediately into liquid N_2, where the disc sets were stored before analysis.

Extraction of the metabolites and pretreatment of the extracts on polyvinylpyrrolidone and DEAE-cellulose columns followed the procedure of Macnicol (16). A proportion (60%) of the pyruvate partitions into the ether extract; it was recovered by three extractions with 5 ml of 0.25 M triethylammonium chloride buffer, pH 7.5. Prior to assay the ether was removed by bubbling with N_2. For the analysis of glycolytic intermediates in the DEAE-cellulose eluate, blank fluorescence and inhibition of coupling enzymes were markedly reduced by additional pretreatment with charcoal. Two milliliters of eluate were set aside for analysis of adenine nucleotides. To the remaining 4 ml were added 400 mg of dry BDH decolorizing charcoal from which the fines had previously been removed by repeated settling from 50 cm of water for 20 min. After standing for 1 hr in the cold with occasional shaking, the suspension was filtered through a small disc of Whatman No. 542 paper, the charcoal was washed with small aliquots of water, and the filtrate was made up to 5 ml.

The metabolites, except for P_i, were assayed using an Eppendorf fluorimeter with ancillary equipment as described earlier (16). Analysis of glycolytic intermediates followed the methods of Maitra and Estabrook (18) with slight modifications. Adenine nucleotides and citrate were assayed as previously described (16).

RESULTS

Leaf excision was followed after 1.5 days by a strong rise in respiration and rapid yellowing (Fig. 1). After 3 days, when the initial rate had been doubled, the respiratory drift showed a gradual fall accompanied by necrosis of the lamina. This began at the tip and edges and proceeded inwards, until by day 7 most of the lamina was brown. From this time onwards withering of the lamina made expression of metabolite levels on an area basis only semiquantitative.

During the respiratory drift the leaves were sampled daily for metabolite analysis by removing discs according to a scheme based on random permutations, so that only about 1% of the total leaf area was removed at any one time. Preliminary data on respiration rate indicate that with this interval between sampling times the disturbance to the rest of the leaf lamina is insignificant. The sampling error was estimated by punching out all the disc sets at once from detached leaves at various stages of yellowing. The coefficient of variation of respiration rate and of chlorophyll content varied between 3 and 5%.

ATP level (Fig. 2) followed a curve remarkably similar to that of the respiratory drift; at the peak the initial level was more than doubled. The changes in ADP and AMP were scarcely significant except for a late rise in AMP level.

Figure 3 shows the changes in levels of glycolytic intermediates relative to their initial levels. Coincident with the respiratory rise at day 1 to 2 there were marked rises in hexose phos-

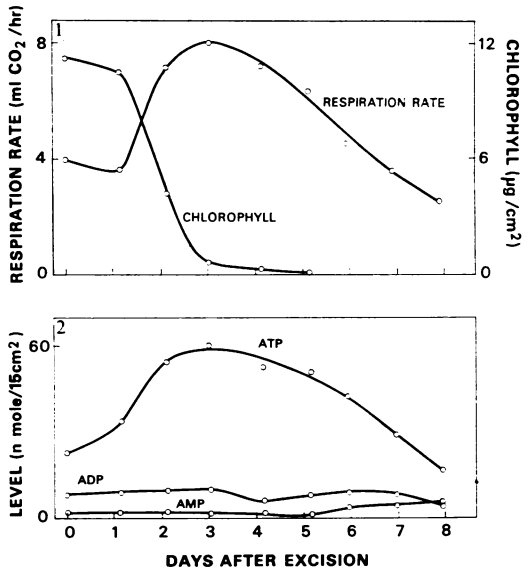

FIG. 1. Drift in respiration rate (whole leaf basis) and chlorophyll content of excised tobacco leaf at 25 C in dark. (Note that in this and the other figures the time scale does not include an initial 3-hr dark equilibration period).

FIG. 2. Adenine nucleotide levels in leaf of Figure 1.

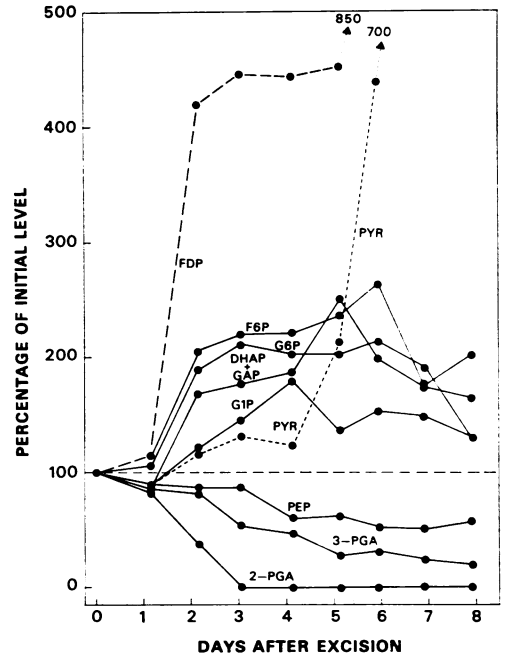

FIG. 3. Levels of glycolytic intermediates in leaf of Figure 1, expressed as percentage of level at day 0. (Actual levels at day 0 in nmoles/15 cm² were: G1P, 8.4; G6P, 62; F6P, 12.6; FDP, 0.4; DHAP + GAP, 5.3; 3-PGA, 23.0; 2-PGA, 3.2; PEP, 7.7; PYR, 22).

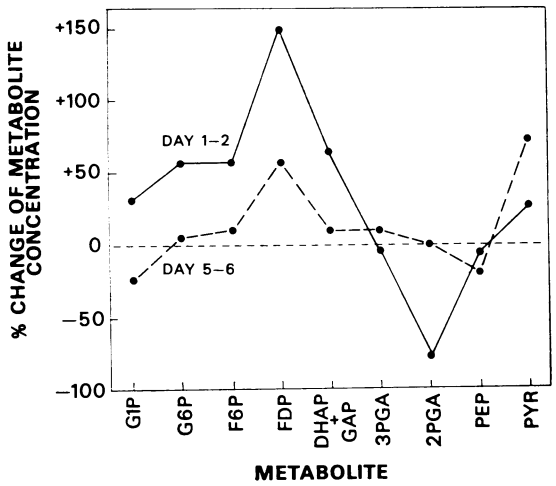

FIG. 4. Crossover plot of data in Figure 3 showing changes between days 1 to 2 (solid line) and 5 to 6 (dashed line). Per cent change of metabolite concentration (C) between time t_1 and t_2 = 100 $\times (C_{t2} - C_{t1})/0.5(C_{t2} + C_{t1})$.

phates, triose phosphates, and PYR[1], while phosphoglycerates and PEP either fell or did not change. After the other hexose phosphates had leveled off, G1P continued to rise up to day 5; in other leaves G1P leveled off earlier. There was a second, steep rise in PYR at day 4 to 5 and in FDP at day 5 to 6. When the data were represented as crossover plots (11), a reverse crossover between triose phosphates and 3-PGA appeared at day 1 to 2 and a forward crossover between PEP and PYR at days 1 to 2 and 5 to 6 (Fig. 4).

DISCUSSION

The prompt yellowing and climacteric rise in respiration rate shown by the leaves used in this study are probably related to the fact that they were deliberately picked at a stage of maturity (physiological age) subjectively similar to that of "ripe" field-grown leaves (21). Their chlorophyll content (when excised) of 10 to 15 $\mu g/cm^2$ had dropped from a content of about 30 $\mu g/cm^2$ at full expansion. It appears that the post-climacteric fall in respiration rate is due to progressive necrosis of the leaf cells; rough calculation based on non-necrotic area suggests that prior to necrosis the yellow cells respire at a constant high rate.

The marked rise in ATP level, unaccompanied by compensating falls in ADP or AMP, indicates considerable synthesis of phosphorylated adenosine. A similar situation was found by Rowan *et al.* (26) in the ripening cantaloupe. It can also be seen that the energy charge (4) of the adenylate system, (2ATP + ADP)/2(ATP + ADP + AMP), over the whole respiratory peak was greater than the initial value. This fact, together with the orderly nature of the changes in glycolytic intermediates, which are very labile to attack by hydrolytic enzymes, indicates significant retention of cellular organization during yellowing. Recent ultrastructural studies (25) show that, despite rapid degeneration of chloroplasts and lysis of the tonoplast, apparently intact mitochondria, ribosomes, and an intact plasmalemma persist in yellow cells of detached *Nicotiana glutinosa* leaves.

In the trends of levels of glycolytic intermediates with time, two phases are distinguishable, both beginning with forward (depletion followed by accumulation) crossovers between PEP and PYR, at days 1 to 2 and 5 to 6, respectively. The first phase also shows a reverse crossover (accumulation followed by depletion) between triose phosphates and 3-PGA. Of these only the first PEP/PYR crossover satisfies the requirements of the theory (forward crossover during increasing flux; Ref. 8), in locating a site of control at pyruvate kinase. Although glycolytic flux was not measured, the doubling of respiration rate, rapid carbohydrate breakdown (13), and development of DNP-resistant $^{32}P_i$ esterification (17) strongly suggests that the flux increased during the climacteric rise. The rather small PEP depletion (6%) involved in this crossover may result from a temporary buffering effect of the reversible reactions between phosphoglycerate kinase and pyruvate kinase, as recently discussed by Adams and Rowan (1). Alternatively, the PEP level may be maintained by nonglycolytic reactions.

Since FDP is thought to be the main *in vivo* modulator of pyruvate kinase activity in liver (28) and yeast (29), the large rise in FDP appears a likely cause of activation of the enzyme in the yellowing leaf. The only higher plant pyruvate kinase thus far reported on, that of *Lemna* (24), was not activated by FDP. Retention of allosteric properties by such enzymes may, however, require stringent control of extraction conditions.

Use of the crossover theorem in the present situation suffers from potential limitations (27). The existence of branch points to other pathways means that the levels of such intermediates would be affected by any flux through these pathways as well as by the glycolytic flux. Thus the level of G6P would be depleted by operation of the oxidative pentose phosphate pathway and that of PEP by dark CO_2 fixation via PEP carboxylase. There is, in fact, evidence that detachment induces stimulation of the oxidative pentose phosphate pathway (30). Another limitation to interpretation is the long time scale of the experiments, which increases the possibility of intermediate steady states that are not detected, as well as entry of metabolites or even enzymes from other pools such as the chloroplast. In the yellowing leaf the latter appears a real possibility. It would be desirable to obtain confirmation of a control function for pyruvate kinase by comparison of the apparent equilibrium constant *in vivo* with the true constant determined *in vitro*.

The striking accumulation of glycolytic intermediates down to triose phosphates and the depletion of phosphoglycerates and PEP at day 1 to 2 suggest inhibition of glyceraldehyde-3-P dehydrogenase or phosphoglycerate kinase. Although the data do not permit a choice between these alternatives, a plausible hypothesis can be based on rate limitation at the glyceraldehyde-3-P dehydrogenase step. It has been found (15) that a NAD-specific glyceraldehyde-3-P dehydrogenase occurs in both chloroplasts and cytoplasm, whereas a NADP-specific enzyme is confined to the chloroplasts. The latter enzyme, according to the work of Müller *et al.* (22), will be converted into a NAD-specific form when the leaves are placed in the dark. Mishra and Waygood (20) showed a marked fall in NAD/NADH ratio in detached wheat leaves during dark periods. On the assumption that this also occurs in detached tobacco leaves, both NAD-specific enzymes will become inhibited, thus restricting glycolytic flux. On the other hand, phosphoglycerate kinase does not appear to function as a control point in glycolytic systems (27), although analytical difficulties associated with the instability of 1,3-diphosphoglycerate (23) may contribute to this situation.

The steep rise in PYR level after the climacteric peak could

[1] Abbreviations: G1P: glucose 1-phosphate; G6P: glucose 6-phosphate; F6P: fructose 6-phosphate; DHAP: dihydroxyacetone phosphate; GAP: glyceraldehyde 3-phosphate; 3-PGA: 3-phosphoglycerate; 2-PGA: 2-phosphoglycerate; PEP: phosphoenolpyruvate; PYR: pyruvate; DNP: 2,4-dinitrophenol.

well be due to breakdown of the mitochondrial oxidative system, particularly since it is accompanied by a rise in citrate level (unpublished data). Neither the early nor the late rise in FDP level was accompanied by a crossover with F6P, so that phosphofructokinase activation is unlikely to be involved. This contrasts with the situation in the ripening tomato (7).

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