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Metabolic Regulation in the Senescing Tobacco Leaf

I. CHANGES IN PATTERN OF ^{32}P INCORPORATION INTO LEAF DISC METABOLITES[1]

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P. K. MACNICOL,[2] R. E. YOUNG,[3] AND J. B. BIALE
Department of Botanical Sciences, University of California, Los Angeles, California 90024

ABSTRACT

Changes in phosphate metabolism were explored in discs from tobacco (*Nicotiana tabacum*) leaves of three contrasting types: green leaves which were fully expanded and attached to the plant, leaves which had yellowed following excision and dark starvation, and leaves which had yellowed while attached to the plant. 2,4-Dinitrophenol at 10^{-5}M stimulated the respiration rate of discs from green and yellow-detached leaves only slightly, but markedly stimulated that of discs from yellow-attached leaves. Following a 10-minute uptake period the incorporation of ^{32}P-orthophosphate into phosphate esters and lipids of discs from yellow-detached leaves was resistant to 2,4-dinitrophenol, whereas in discs from green and yellow-attached leaves it was inhibited by 2,4-dinitrophenol. Incorporation into a salt-soluble fraction containing unidentified nucleotide material showed converse behavior in that it was stimulated by 2,4-dinitrophenol in discs from green and yellow-attached leaves; in discs from yellow-detached leaves it was resistant to 2,4-dinitrophenol. In discs from yellow-detached and yellow-attached leaves there was a shift in the labeling pattern of phosphate esters toward increased label in hexose phosphates at the expense of adenine nucleotides, 3-phosphoglycerate, and phosphoenolpyruvate. It is concluded that incorporation into phosphate esters in discs from yellow-detached leaves is by substrate level phosphorylation coupled to enhanced aerobic glycolysis. In discs from yellow-attached leaves, on the other hand, incorporation depends on oxidation phosphorylation, and it is suggested that the shift in labeling pattern is caused by senescence-induced changes in activity of glycolytic enzymes.

Leaf senescence plays a significant role in determining the composition of many agricultural products. Thus, in edible plant organs such as leaves, seeds, and roots the pattern of accumulation of storage proteins and carbohydrates will be affected by age-dependent changes in precursor pools in the leaf (5, 24), which in turn reflect a changing balance of metabolic pathways. In the case of tobacco, changes in the attached leaf during ripening and in the detached leaf during curing both exert a profound influence on the quality of the final product (8, 21).

The sequential yellowing and necrosis of the lower leaves of an annual plant such as tobacco are closely related to remobilization of nutrients for translocation to the apex (12, 31). There are many indications that this senescence of the leaf cells, as well as the over-all senescence of the plant, is under genetic control (16, 20) but is also subject to "triggering" by external and internal signals such as shading (12), photoperiodic regime (14), and changed sink-source relationships (27).

Although the over-all process of leaf yellowing is catabolic, as witnessed by the loss of chlorophyll, protein, and RNA (*e.g.*, 11, 13, 32), there is evidence for synthesis of amides (34), pigments and lipids (10), and enzymes (7, 19). Any remobilization of leaf components will require a supply of ATP. It is, in fact, well established that the dark respiration rate usually rises during yellowing (1, 6, 13). These considerations suggest an important interaction between the orderly events of senescence and the control of energy generation.

The more rapid and pronounced nature of the changes in the detached leaf, coupled with their broad similarity to those in the attached leaf, has led to the widespread assumption (*e.g.*, 19, 32) that the detached leaf can serve as an experimental model for senescence in the attached leaf. Several observations suggest, however, that the two situations are different. These include the apparent inability of the detached leaf to synthesize particular species of RNA (15) as well as differences in patterns of nuclease accumulation (33) and ultrastructural breakdown (25).

In spite of the likely requirement for respiratory energy by the integrated process of leaf senescence, data are lacking on the respiratory metabolism of both attached and detached yellowing leaves. The present paper examines the esterification of $^{32}P_i$ into respiratory metabolites by discs from such leaves, using as a point of reference discs from nonsenescent green leaves. The uncoupler DNP is employed to gauge to what extent ATP formation is coupled to respiratory electron transport. The results reveal marked differences between yellow and green leaves, on the one hand, and between attached and detached yellow leaves, on the other, which are indicative of altered metabolic control. A companion paper (18) reports changes in endogenous metabolite levels in the yellowing detached leaf during its "climacteric rise" in respiration and their interpretation in terms of control points.

MATERIALS AND METHODS

Plant Materials. Tobacco plants (*Nicotiana tabacum* L., cv. Virginia Gold) were grown in 3½-gallon drums of clay-loam soil in a smog-screened glasshouse at 32 C (day) and 24 C (night). Supplementary nitrogen was given as 0.5 to 1.0 g of

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[2] Present address: Division of Plant Industry, Commonwealth Scientific and Industrial Research Organization, Canberra, A.C.T. 2601, Australia.

[3] Present address: Department of Plant Science, University of California, Riverside, Calif. 92502.

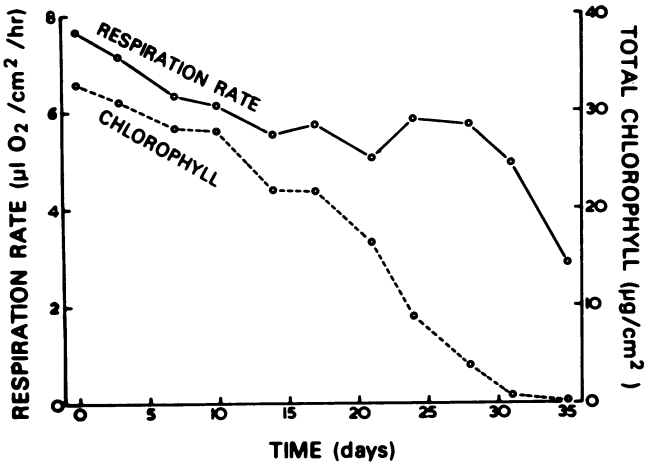

FIG. 1. Changes in the dark respiration rate and chlorophyll content of an attached tobacco leaf subsequent to full expansion, as determined on disc samples.

NH_4NO_3, weekly until flowering commenced. Large basal leaves were picked from positions 8 to 10, counting as 1 the first leaf above the cotyledons. *Green*, fully expanded leaves were harvested from plants in bud. *Yellow-detached* leaves were obtained by allowing green leaves to stand for 5 to 6 days with their petioles in water in the dark at 25 C, until chlorophyll disappearance was essentially complete. *Yellow-attached* leaves were harvested from plants which had nearly finished flowering. Green and yellow-attached leaves were allowed to equilibrate in the dark with their petioles in water for 3 hr prior to sampling.

Sampling. Leaf discs 1 cm^2 in area were removed by a sharp punch under dim green light according to either of two sampling methods. For experiments measuring respiration rate, 8 sets of 24 discs were taken using a scheme (unpublished) based on random permutations of 8. This method gave a coefficient of variation between replicate sets of about 3%. For the $^{32}P_i$ incorporation experiments, 2 sets of 50 discs were taken by a "cluster-pair" method, so that one member of each pair went into one set (DNP)[4] and the other into the other set (control).

Measurement of Respiration Rate. Oxygen uptake was measured by the Warburg technique at 25 C. For experiments not involving DNP the discs were supported on a thin layer of glass wool moistened with 0.1 ml of water. For examination of the effect of DNP on respiration rate the discs were placed lower side downwards on 5 ml of 20 mM Na-K phosphate buffer, pH 5.5, containing DNP at the required concentration and vacuum-infiltrated twice at 68 cm Hg. The flasks were shaken at 90 cycles/min.

Determination of Chlorophyll Content. The sets of leaf discs were recovered from the Warburg flasks and extracted with 80% acetone; chlorophyll was determined in the supernatants by the method of Bruinsma (4).

Incorporation of $^{32}P_i$. The discs, separated by small glass beads, were quickly threaded in groups of 10 onto thin stainless steel wires. One set of 50 was placed in 25 ml of 10^{-5} M DNP in 0.1 mM Na phosphate buffer, pH 5.5, and the other in buffer only; 150-ml beakers were used. Following vacuum infiltration of the discs (twice at 68 cm Hg), the beakers were shaken at 95 cycles/min in a Dubnoff incubator in the dark at 25 C for 40 min to ensure penetration of the DNP. Radioactive orthophosphate (about 0.5 mc in 100 µl of 1 mM H_3PO_4) was then added, and shaking was continued. After a 10-min uptake period the discs were washed in 500 ml of ice-water for 3 min (longer washing did not remove any more radioactivity), briefly drained on filter paper, and dropped (less wires) into 10 ml of boiling methanol-chloroform (2:1) mixture in a tube fitted with a "cold finger" condenser. Boiling was continued for 3 min.

Extraction and Chromatography of Labeled Phosphate Esters. The Bieleski-Young procedure (3) was used, with the modification that the methanol-$CHCl_3$-H_2O ratio was 17:10:3 instead of 12:5:3. Following extraction with methanol-$CHCl_3$-H_2O (2 ml, 5 times) the disc residue on the filter was extracted by homogenization with cold 0.2 N formic acid in 20% ethanol (5 ml, 3 times, with 5 ml washings) and stored at −15 C pending extraction with NaCl. After making the methanol-$CHCl_3$-H_2O extract biphasic by addition of water (5 ml) and chloroform (5 ml), the aqueous phase was added to the formic acid-ethanol extract to give the phosphate ester fraction. The chloroform-rich phase constituted the lipid fraction. The phosphate ester fraction was rotary-evaporated to dryness at 25 C, dissolved in 3 ml of water, and freed from cations and neutral compounds by passing through columns of Cellex-P (1 g) and Cellex-D (2 g; both from BioRad). After rotary evaporation of the ammonium bicarbonate eluate the residue was dissolved in 0.5 ml of water, and a 50-µl aliquot was subjected to two-dimensional paper chromatography in *n*-propanol-ammonia-water (6:3:1) followed by *n*-propyl acetate-formic acid-water (11:5:3) (ref. 3). Radioautograms were then made to locate the labeled phosphate esters. The spots were cut out and placed in planchets, and their radioactivity was determined with a Nuclear-Chicago thin window gas flow counter. The identity of these compounds was checked by chromatographing standards together with labeled leaf extracts and confirming coincidence of radioactivity with phosphate detection of the standards by the modified Axelrod-Bandurski method (3). The identity of FDP was confirmed by treatment with aldolase, triosephosphate isomerase, and α-glycerolphosphate dehydrogenase followed by electrophoretic separation (3), showing coincidence of labeled with standard α-glycerolphosphate.

With the purpose of extracting nucleic acids from the disc residue, it was homogenized in 7 ml of 1 M NaCl, and the brei was heated at 70 C for 30 min before being centrifuged at 10,000*g*. This extraction was repeated twice.

RESULTS

The drifts in dark respiration rate and chlorophyll content of the senescing attached tobacco leaf, measured using disc samples, are shown in Figure 1. A temporary rise in respiration rate of about 10 days' duration appears as a shoulder in the downward drift, coinciding with a final accelerated disappearance of chlorophyll. As shown in the next paper (18), the detached leaf exhibits a more striking rise in respiration rate, the "climacteric peak," over a shorter period. The leaves used in the present study showed a very pronounced preclimacteric minimum, so that the rate at the peak was somewhat lower than the initial rate. This contrast with the respiratory drift of the material grown in Australia (18) presumably relates to the difference in growing conditions.

Subsequent experiments were performed with discs from leaves in contrasting states of senescence: green, yellow-detached and yellow-attached (see "materials and methods"). The penetration of added DNP into discs vacuum-infiltrated with buffer required 30 to 40 min as revealed by a bend in the plot of respiration against time. This may reflect the time

[4] Abbreviations: DNP: 2,4-dinitrophenol; G6P: glucose 6-phosphate; 3-PGA: 3-phosphoglycerate; PEP: phosphoenolpyruvate; FDP: fructose 1,6-diphosphate; FCP: fructose 6-phosphate.

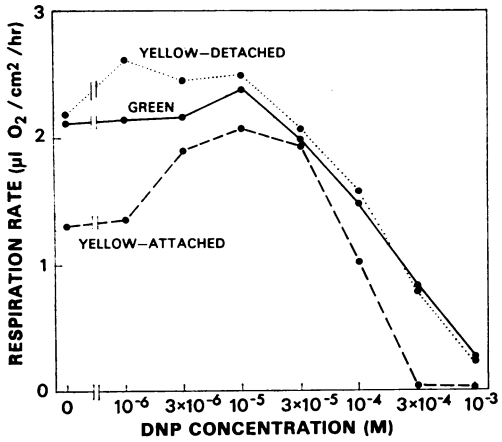

FIG. 2. Effect of DNP concentration on dark respiration rate of discs from green, yellow-detached, and yellow-attached tobacco leaves.

required for permeation through the leaf cell plasma membrane. Thereafter the altered respiration rate was linear for at least 1 hr at DNP concentrations up to 10^{-4} M but fell off progressively at higher concentrations. Figure 2 shows the relationship of respiration rate to DNP concentration. Discs from all three leaf types exhibited maximal stimulation of oxygen uptake at 10^{-5} M DNP; in the case of yellow-detached leaves a further peak at 10^{-6} M, the significance of which is unclear, was often observed. Discs from yellow-attached leaves showed much greater stimulation over the buffer control (likewise vacuum-infiltrated) and more inhibition by higher DNP concentrations.

Exposure of the leaf discs to $^{32}P_i$ in the dark resulted in linear uptake of radioactivity for at least 1 hr if they had been vacuum-infiltrated; otherwise the rate of uptake fell off after 10 min. Of the radioactivity taken up during the normal 10-min incubation period followed by a 3-min rinse in ice-water, less than 5% could be removed in the cold with strong Na phosphate solution (10 mM, pH 6.8) during a 30-min period. The contribution of simple exchange or of "free space" phosphate to the uptake is therefore negligible.

Extraction of the labeled discs yielded a phosphate ester fraction (including P_i), a lipid fraction, and a disc residue. The phosphate esters were separated from P_i by chromatography.

Further extraction of the disc residue with hot 1 M NaCl (final pH approximately 6), with the aim of releasing nucleic acids (23), solubilized approximately 90% of the remaining radioactivity. The UV spectrum of this extract showed a marked shoulder at 260 nm superimposed on a heavy end-absorption. However, the fact that this material was not precipitated by ethanol and dialyzed through a Visking membrane into water was not consistent with its being nucleic acid. It was tenaciously adsorbed to acid-washed Norit A (28), only 20 to 30% being eluted by 10% aqueous pyridine at 37 C. When the eluate was chromatographed on paper in isobutyric acid-ammonia-aqueous EDTA (28) with and without marker $^{32}P_i$, the radioactivity ran as a weakly UV-quenching streak with lower mobility than P_i.

Table I shows the partition of ^{32}P among these fractions in the presence and absence of 10^{-5} M DNP. This concentration of DNP, which induced maximal respiratory stimulation in all three types of discs (Fig. 2), was found to inhibit strongly $^{32}P_i$ incorporation into phosphate esters and lipids in discs from green and yellow-attached leaves but not from yellow-detached leaves. Incorporation into the NaCl-soluble fraction was markedly stimulated by DNP in discs from green and yellow-attached leaves but not from yellow-detached leaves.

Table II shows the distribution of label among phosphate esters of green discs, in the absence of DNP, resolved by two-dimensional paper chromatography. There is a high recovery

Table II. *Incorporation of $^{32}P_i$ into Phosphate Esters of Green Tobacco Leaf Discs*

Metabolite	Percentage of Total Radioactivity on Chromatogram
ATP	38.3
G6P	13.3
ADP	12.4
3-PGA	8.9
Uridine 5′-triphosphate	7.0
Uridine 5′-diphosphate	1.8
G1P	1.7
F6P	1.7
Guanosine 5′-triphosphate	1.6
PEP	1.5
Guanosine 5′-diphosphate	0.8
α-Glycerolphosphate	0.8
Uridine diphosphoglucose	0.6
FDP	0.4
Pyrophosphate	0.2
Unidentified	9.0
Total	100.0

Table I. *Effect of 10 µM DNP on Incorporation of $^{32}P_i$ into Various Fractions of Discs from Tobacco Leaves in Three Contrasting States of Senescence*

Incorporation (−DNP and +DNP) expressed as cpm × 10^{-3}/50 discs·mc offered.

Fraction	Green Leaf			Yellow-Detached Leaf			Yellow-Attached Leaf		
	−DNP	+DNP	+DNP/−DNP × 100	−DNP	+DNP	+DNP/−DNP × 100	−DNP	+DNP	+DNP/−DNP × 100
Inorganic phosphate	9,070	11,900	131	7,700	8,560	110	6,180	6,820	110
Phosphate esters[1]	1,240	253	20	1,490	1,330	89	831	278	33
Lipid (chloroform layer)	152	51	34	162	164	101	92	22	24
NaCl extract of disc residue	1,150	4,340	380	552	576	104	228	620	270
Final residue	120	604	500	65	67	103	71	82	116
Total	11,730	17,150	146	10,040	10,700	107	7,400	7,800	105

[1] For details of components of this fraction see Table II.

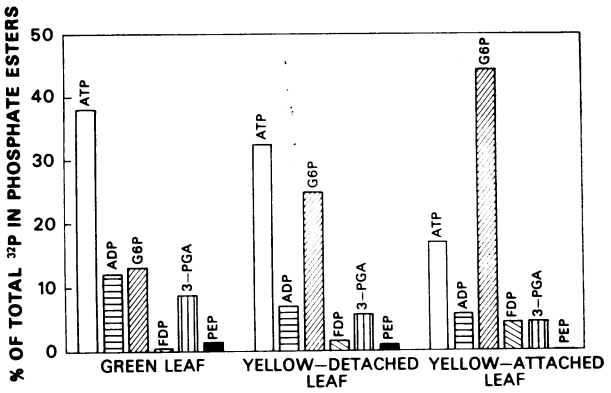

FIG. 3. ^{32}P labeling patterns of respiratory metabolites, separated by two-dimensional paper chromatography, in discs from green, yellow-detached, and yellow-attached tobacco leaves.

Table III. *Effect of 10 μM DNP on Incorporation of $^{32}P_i$ into Metabolites by Discs from Tobacco Leaves in Three Contrasting States of Senescence*

	Green Leaf	Yellow-Detached Leaf	Yellow-Attached Leaf
	% of value for corresponding control discs		
ATP	15	86	21
ADP	35	102	48
G6P	26	88	35
FDP	28	200	100
3-PGA	10	66	24
PEP	7	61	...[1]

[1] Activity on chromatogram insufficient for accurate counting.

of radioactivity in known phosphate esters, half of this ^{32}P being in ATP and ADP alone. Qualitatively the spectrum of labeled metabolites was the same for the three types of leaves.

In contrast to this qualitative similarity, the labeling patterns exhibited some striking quantitative differences. Figure 3 shows data for six metabolites of respiration in the three types of leaf discs. In both types of yellow discs much more ^{32}P enters G6P; in discs from yellow-attached leaves it dominates the pattern. In both cases this rise is accompanied by an increase in the labeling of FDP and a decrease in the labeling of ATP, ADP, 3-PGA, and PEP.

The effect of 10^{-5} M DNP on these labeling patterns is shown in Table III. Discs from green and yellow-attached leaves were similar in the severe inhibition of labeling of ATP, ADP, G6P, and 3-PGA, but in discs from yellow-detached leaves the inhibition was relatively slight. Interestingly, the labeling of FDP was inhibited by DNP in discs from green leaves, unaffected in discs from yellow-attached leaves, and stimulated in discs from yellow-detached leaves.

DISCUSSION

This paper explores similarities and differences in the phosphate metabolism of discs from leaves chosen to differ maximally in type and degree of senescence: green, fully expanded leaves; detached leaves that had yellowed during starvation in the dark; and leaves that had yellowed while attached to the plant. All of these leaves were picked from the same stalk positions, thus eliminating effects due to senescence of the apical meristem (26). Although respiratory measurements were not made on the same leaves from which discs were taken, it can safely be assumed that the yellow-detached leaves were close to the climacteric peak (18). In the case of the yellow-attached leaves, however, this assumption does not hold owing to the transitory nature of the respiratory rise.

Treatment with a range of concentrations of DNP caused a typical biphasic response of respiration rate, with stimulation up to 10^{-5} M and inhibition at higher concentrations. Discs from yellow-attached leaves showed markedly greater stimulation than from green or yellow-detached leaves. Such a biphasic response is also shown by isolated mitochondria, where there is evidence (30) that the stimulation and inhibition result from different sites of action of the uncoupler. In isolated plant mitochondria the extent of DNP stimulation of O_2 uptake is apparently limited by a DNP-insensitive control site (22) as well as by the availability of pyruvate from glycolysis (9). Loss of this site during leaf senescence would increase the potential for stimulation.

Exposure of discs to $^{32}P_i$ for a 10-min period resulted in uptake which did not differ markedly between the leaf types. The fact that vacuum infiltration was required for uptake to be linear with time suggests that the uptake and hence presumably esterification of phosphate are not due primarily to the peripheral cells of the discs. This agrees with recent findings (17) using wheat leaf segments.

Fractionation of the label in the discs revealed two interesting effects of DNP. One was the DNP resistance of incorporation into phosphate esters and lipids by discs from yellow-detached leaves, indicating lack of requirement for respiratory chain phosphorylation. The other was the inverse behavior toward DNP shown by the phosphate esters and phospholipids (inhibited incorporation), on the one hand, and the NaCl fraction (stimulated incorporation), on the other, in discs from both green and yellow-attached leaves. This suggests that in these tissues incorporation into the NaCl fraction is linked to substrate level phosphorylation which is accelerated in the presence of DNP. Alternatively, it may reflect changed compartmentalization of ATP formation in the presence of DNP.

Hot NaCl extraction has been shown to release nucleic acid from a variety of animal and plant tissues (23). Surprisingly, the label in the NaCl extract of tobacco leaf discs proved to be dialyzable and was not precipitated by ethanol. Its tight adsorption to charcoal and chromatographic mobility indicate that it is not polyphosphate or difficulty-extractable P_i. The nature of this fraction, which appears to contain nucleotide material, is under investigation; the possibility that it is formed by nuclease attack during tissue inactivation has not been eliminated. Yellow leaf discs of both types seem to have much reduced capacity for its synthesis compared with green discs.

The pattern of labeling in glycolytic metabolites in the absence of DNP showed a quantitative shift toward hexose phosphates, particularly FDP, in discs from both types of yellow leaves, at the expense of adenine nucleotides, 3-PGA, and PEP. The kinetics of metabolite labeling were not examined. Bieleski and Laties (2) found, after a 30-sec pulse of $^{32}P_i$, that the half-time for labeling nucleoside triphosphates in unaged potato tuber slices was <1 min, but that for hexose phosphates, 3-PGA, and PEP it was 5 to 6.5 min. If similar kinetics obtained in leaf discs, the observed metabolite patterns from a 10-min pulse would represent relative pool size rather than rate of labeling. Data presented elsewhere (18) suggest that this is so, at least for detached leaves. It is noteworthy that a rise in FDP level accompanied by a fall in PEP is characteristic of increased glycolytic flux in both higher plants and yeast (29).

The results strongly suggest that in yellow-detached leaves

the climacteric rise is maintained by a large increase in aerobic glycolysis. This is supported by data (unpublished) showing a rise in respiratory quotient and in citrate level during the climacteric peak. The following paper (18) presents evidence for activation of pyruvate kinase and exposure of a rate limitation at the glyceraldehyde 3-phosphate dehydrogenase step.

With yellow-attached leaves the shift in labeling pattern of phosphate esters was still more pronounced than with yellow-detached leaves, yet uptake into individual phosphate esters, with the exception of FDP, was inhibited by DNP. Taken together with the marked DNP stimulation of respiration rate, these facts suggest that natural senescence is accompanied not by increased aerobic glycolysis but rather by changes in the relative activities of glycolytic enzymes. This aspect is under investigation.

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