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RAPID DECARBOXYLATION OF THE PRODUCTS OF DARK FIXATION OF CO_2 IN ROOTS OF *PISUM* AND *PLANTAGO*

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Key Word Index—*Plantago major*; *P. lanceolata*; Plantaginaceae; plantain; *Pisum sativum*; Leguminosae; pea; roots; dark fixation of CO_2.

Abstract—Seedlings of *Pisum sativum* and excised roots of *Plantago major* and *P. lanceolata* were given, in the dark, a pulse of $^{14}CO_2$ in air followed by a chase in $^{12}CO_2$ in air. A very substantial proportion of the ^{14}C fixed into organic compounds in the pulse was lost from the tissues in the chase. The activity of NAD malic enzyme in extracts of roots of all three species exceeded their rate of respiration. Azide, 2-*n*-butylmalonate, and salicylhydroxamic acid each inhibited CO_2 fixation by excised roots of pea. The first two compounds inhibited respiratory gas exchange, but the third stimulated it. Arguments are presented for the widespread diversion of phosphoenolpyruvate from glycolysis to oxaloacetate and thence to malate in the cytosol followed by transport of the malate into the mitochondria for conversion to pyruvate via NAD malic enzyme. No differences, in the above respects, were found between the two species of *Plantago*.

INTRODUCTION

In the dark, clubs of the spadices of thermogenic Araceae readily fix CO_2 into malate that is promptly decarboxylated [1]. This proceeds via phosphoenolpyruvate carboxylase and NAD malate dehydrogenase in the cytosol, followed by transport of the malate into the mitochondrial matrix where it is converted to pyruvate and CO_2 by NAD malic enzyme (EC 1.1.1.39) [2]. The aim of the work reported in the present paper was to discover if the above sequence is a general feature of the respiratory metabolism of non-photosynthetic tissues of plants.

RESULTS AND DISCUSSION

To determine whether rapid release of recently fixed carbon was a widespread feature of non-photosynthetic tissues of plants, we exposed pea seedlings, and the roots of *Plantago major* and *P. lanceolata* to $^{14}CO_2$ in the dark and observed whether ^{14}C fixed into organic compounds was lost in a subsequent chase in $^{12}CO_2$. The pea seedlings were dissected into hypocotyl, root cortex, stele and root apex before analysis so that we could see how each tissue behaved. The results obtained for the different tissues of the pea seedling and the roots of the two species of plantain are sufficiently similar to be discussed together (Table 1). As expected there was appreciable fixation of ^{14}C and nearly all of it was recovered in the water-soluble compounds at the end of the pulse. During the chase a small proportion of the ^{14}C in the soluble fraction was incorporated into insoluble material. It is likely that this reflects the use of amino acids, labelled in the pulse, for protein synthesis [3]. However, the major change that occurred during the chase was the substantial loss of ^{14}C from the tissues. This is comparable to that found in the clubs of *Arum* [1]. The rapidity of the loss is illustrated by data for excised roots of pea (Fig. 1). It is clear that rapid loss of recently fixed carbon is a widespread phenomenon and not a specialized feature of thermogenic tissues.

There is adequate evidence for the widespread occurrence of substantial activities of both phosphoenolpyruvate carboxylase and NAD malate dehydrogenase [4–6]. As this is not so for NAD malic enzyme, we determined whether plantain and pea roots contained enough of it to make significant contributions to respiration. The difficulties in assaying this enzyme have been discussed [2] and were taken into account. For the plantain roots we optimized the concentration of each component, and the pH of the extraction medium and the assay mixture. $MnCl_2$, $MnSO_4$, $MgSO_4$ and $MgCl_2$ were compared in the extraction medium and the assay mixture. After optimization of the latter, Mg^{2+} did not give higher activity than Mn^{2+}: nor with Mn^{2+} was activity with SO_4^{2-} higher than that with Cl^-. When NAD reduction and pyruvate accumulation were measured in the same reaction mixtures the latter was 107–123% of the former. To check against loss of activity during extraction we prepared, for each test, a sample of roots of *P. major*, a sample of roots of *P. lanceolata*, and a sample that contained equal weights of the roots of both species. The activities in the mixtures were within 9% of the values predicted from the measurements made on the separate components of the mixtures. This suggests that the extracts did not cause substantial inhibition of the enzyme. Consequently, we propose that the following values reflect the maximum catalytic activities of NAD malic enzyme in the apical 4 cm of roots of *P. major* and *P. lanceolata*, respectively: 1.47 ± 0.04, 1.40 ± 0.03 μmol NAD reduced min^{-1} g^{-1} fr wt. Each value is the mean ± s.e.m. of estimates from six extracts. Extracts of the

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Table 1. Labelling of plantain and pea tissues in the dark during pulse in $^{14}CO_2$ and chase in $^{12}CO_2$

Tissue		^{14}C fixed (dpm/mg fr. wt)	
		Soluble compounds	Insoluble compounds
Roots of *Plantago lanceolata*	Pulse	14.96±2.23	2.27±0.81
	Pulse and chase	6.55±0.65	2.82±0.72
Roots of *Plantago major*	Pulse	20.74±1.58	2.17±0.19
	Pulse and chase	6.33±0.67	3.27±0.07
Seedlings of *Pisum sativum*			
Apical 6 mm of root	Pulse	224	32
	Pulse and chase	93	42
Stele 6–52 mm from root apex	Pulse	224	21
	Pulse and chase	98	35
Cortex 6–52 mm from root apex	Pulse	95	4.7
	Pulse and chase	55	6.0
Epicotyl, basal 20 mm	Pulse	97	4.2
	Pulse and chase	64	9.3

For plantains, pulse and chase were each of 150 min and values are means ± s.e.m. from three samples. For peas, pulse was 46 min and chase 120 min: values are for one experiment but similar data were obtained in three other experiments with different lengths of pulse and different specific activity $^{14}CO_2$.

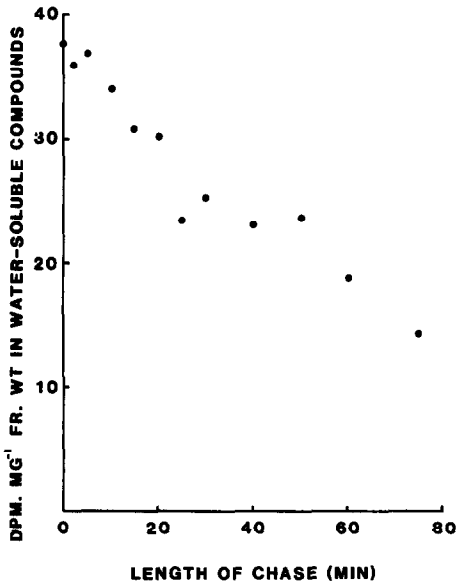

Fig. 1. Loss of ^{14}C from the water-soluble components of the excised apical 24 mm of the roots of *Pisum sativum* after exposure to $^{14}CO_2$ in the dark for 30 min.

apical 2.4 cm of pea roots had an activity of 0.92 μmol min^{-1} g^{-1} fr wt: this is a minimum estimate as the assay mixtures were not optimized. The maximum rates of respiration of excised roots of the plantains were less than 0.24 μmol CO_2 min^{-1} g^{-1} fr wt. The value for pea roots was 0.41 μmol CO_2 min^{-1} g^{-1} fr wt. Thus there is sufficient NAD malic enzyme in the roots of all three species to make very substantial contributions to respiratory metabolism.

If release of the recently fixed carbon involves transport of cytosolic malate into the matrix of the mitochondria for metabolism by NAD malic enzyme, then 2-*n*-butylmalonate would be likely to inhibit CO_2 fixation. This is because 2-*n*-butylmalonate inhibits transport of malate into mitochondria [7] and the resulting accumulation of malate in the cytosol would be expected to inhibit phosphoenolpyruvate carboxylase [8]. 2-*n*-Butylmalonate caused very extensive inhibition of $^{14}CO_2$ fixation by excised roots of peas (Table 2). The concentrations of butylmalonate that almost completely inhibited $^{14}CO_2$ fixation also inhibited respiration (Table 3). These observations implicate phosphoenolpy-

Table 2. Effects of 2-*n*-butylmalonate, salicylhydroxamic acid and azide on labelling by $^{14}CO_2$ of water-soluble compounds of the excised apical 24 mm of roots of *Pisum sativum*

Butylmalonate		Salicylhydroxamic acid		Azide	
concn (mM)	^{14}C fixed (dpm/mg fr. wt)	concn (mM)	^{14}C fixed (dpm/mg fr. wt)	concn (μM)	^{14}C fixed (dpm/mg fr. wt)
0	39.2	0	35.8	0	30.9
2	32.1	0.1	32.2	1.5	26.6
5	25.0	0.2	26.4	5	22.4
10	17.7	0.5	21.0	15	14.8
20	10.4	1.0	17.7	50	0.9
50	1.1	3.0	6.7	150	0.3
100	0 2	6.0	2.8	500	0.1
		12.0	0.5		

Samples of 10 apices (0.2 g fr. wt) were pre-treated with inhibitor for 90 min and then exposed to $^{14}CO_2$ for 30 min.

Table 3. Effects of 2-*n*-butylmalonate, salicylhydroxamic acid and azide on respiration of the excised apical 24 mm of roots of *Pisum sativum*

Inhibitor	Gas exchange (μl/hr per 20 mg dry wt)	
	O_2 uptake	CO_2 production
None	116±4	107±1
Butylmalonate, 50 mM	73±8	68±3
Salicylhydroxamic acid, 6 mM	160±8	165±1
Azide, 0.05 mM	69±6	112±5

Values are means ± s.e.m. from triplicate samples for the period 30–90 min after the addition of inhibitor.

ruvate carboxylase, and transport of cytosolic malate to the mitochondria in the respiration of the roots.

Further evidence of a close relationship between CO_2 fixation and respiration is provided by our observations that CO_2 fixation was readily inhibited by azide and salicylhydroxamic acid (Table 2). Both these compounds could interfere with NADH oxidation and the operation of the Krebs cycle. Evidence that azide did so is provided by the fact that it inhibited oxygen uptake and led to some degree of aerobic fermentation (Table 3). The effects of salicylhydroxamic acid are less straightforward as concentrations that gave over 90% inhibition of CO_2 fixation stimulated both oxygen uptake and CO_2 production.

There is already considerable evidence that a significant proportion of the phosphoenolpyruvate produced in glycolysis is converted to malate via phosphoenolpyruvate carboxylase and malate dehydrogenase [4, 9]. The data in this paper add to this evidence, and together with those from *Arum* [1, 2], strongly suggest that much of the above malate is transported into the mitochondria where it is converted to pyruvate via NAD malic enzyme. The significance of this roundabout route to pyruvate is unlikely to become apparent until more is known of the relative importance of malate and pyruvate transport into mitochondria, and of the re-oxidation of NADH formed in glycolysis.

The roots of the two species of plantain that we have studied have been said to differ in their respiratory efficiency, and part of this difference has been attributed to differences in the contribution of the cyanide-resistant alternative oxidase [10, 11]. Rustin *et al.* [12] have suggested a specific association between the alternative oxidase and NAD malic enzyme. There was a very close similarity between the roots of the two plantains in respect of $^{14}CO_2$ metabolism (Table 1) and activity of NAD malic enzyme. This suggests that, if there is a major difference in the respiratory metabolism of the roots of the plantains, it does not lie in their capacity to convert phosphoenolpyruvate to pyruvate via NAD malic enzyme.

EXPERIMENTAL

Material. Substrates and cofactors were from Boehringer; isotopes from The Radiochemical Centre, Amersham; salicylhydroxamic acid from Aldrich, Gillingham, U.K.; 2-*n*-butylmalonate as in ref. [2]. Peas, *Pisum sativum* L. cv. Kelvedon Wonder were grown as in ref. [13], dissected as in ref. [14] and used when 5 days old. Seeds of *Plantago lanceolata* L. and *Plantago major* L. were collected locally and germinated and grown for 4 weeks in moist sand at 20–28° with natural illumination supplemented with an 18 hr photoperiod of 10 W/m² PAR from Atlas 75/85 W white fluorescent tubes. Next the seedlings were removed from the sand and supported in evenly spaced holes in a lid that covered a bowl (33 × 27 × 12 cm), that contained continuously aerated culture solution; macronutrients [15], micronutrients [16] but at 25% of values quoted. Subsequent growth was at 20° with a 12 hr photoperiod and light at 50 W/m² PAR from 60 cm 20/33 W white Philips fluorescent tubes and 15 cm 30 W tungsten strip lights in a ratio of 6:2. The complete root systems were excised after 16 days (*P. lanceolata*) and 21 days (*P. major*), and used immediately to prepare the experimental samples

Metabolism of $^{14}CO_2$. For exposure of pea seedlings to $^{14}CO_2$, 26 5-day-old seedlings were put on damp cotton wool in a 2 l. flask. The latter was sealed and 50 μCi $^{14}CO_2$ (58.5 Ci/mol) was injected via a self-sealing bung. The flask was kept at 25° in the dark for 46 min. At the end of this pulse 13 of the seedlings were removed, quickly dissected, and killed in boiling 80% (w/v) aq. EtOH. The other 13 seedlings were incubated in air, but otherwise under the same conditions as the pulse, for a chase of 120 min. At the end of the chase the seedlings were dissected and killed as described above. For experiments with excised roots of peas samples (0.2 g fr. wt) of the apical 24 mm of the roots were exposed to $^{14}CO_2$ for 30 min as above except that they were suspended in a flask to which 10 μCi $^{14}CO_2$ (58.5 Ci/mol) were added. For experiments with *Plantago*, samples (150 mg fr. wt) of complete root systems were treated like the excised roots of peas except that 5 μCi $^{14}CO_2$ was introduced into flasks that contained 0.3% (v/v) CO_2 in air and the chase was in 0.3% CO_2 in air. All samples were extracted, successively, in boiling 80%, 50%, 20% aq. EtOH, H_2O and 80% EtOH. The extracts were combined, reduced almost to dryness at 25° and made up to a known volume to give the water-soluble fraction. The extracted residue is the insoluble fraction. The ^{14}C in each fraction was measured as described in ref. [1]. For investigation of the effects of inhibitors on $^{14}CO_2$ fixation, samples of excised roots of pea were pre-treated by incubation for 90 min at 25° in inhibitor in 25.5 mM Na_2HPO_4–12.2 mM citric acid buffer, pH 5.2, then blotted dry and exposed to $^{14}CO_2$ as described above for excised roots.

Enzyme measurements. NAD malic enzyme was measured by following the formation of NADH that was dependent upon the presence of both malate and $MnCl_2$ as described in ref. [2]. Samples, 0.5 g fr. wt, were homogenized with 40 mg insoluble polyvinylpyrrolidone and 70 vol 25 mM Hepes adjusted to pH 7.2 with NaOH, 1.0 mM $MnCl_2$, 10.2 mM dithiothreitol, 0.1% (v/v) Triton X-100. The homogenate was centrifuged at 80 000 *g* for 30 min and the supernatant was assayed at once in a reaction mixture (1 ml) that contained, at pH 6.9, 43 mM MOPS, 22.5 mM malate, 15.5 mM NAD, 0.3 mM EDTA, 0.15 mM CoA, 20.4 mM $MnCl_2$. Extraction was at 2° and assay at 25°. Pyruvate produced in the assay was measured as in ref. [2].

Respiration. Gas exchange was measured manometrically by Warburg's direct method at 25°. Samples, 0.2–0.3 g fr. wt, were suspended in 2.25 ml 25.5 mM Na_2HPO_4–12.2 mM citrate buffer, pH 5.2, and 0.5 ml inhibitor, dissolved in this buffer, was added from the side-arm.

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