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# EFFECTS OF ANAEROBIOSIS ON CARBOHYDRATE OXIDATION BY ROOTS OF *PISUM SATIVUM*

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**Key Word Index**—*Pisum sativum*; Leguminosae; pea; roots; anaerobiosis; fermentation; sugar; enzymes; ethanol; alanine.

**Abstract**—The aim of this work was to discover the effects of anaerobiosis on the breakdown of sugars by the apical 6 mm of the roots of 5-day-old seedlings of *Pisum sativum*. Estimates of the maximum catalytic activities of alcohol dehydrogenase, lactate dehydrogenase, phosphoenolpyruvate carboxylase and NADP-specific malic enzyme showed them to be comparable to that of phosphofructokinase. Metabolism of sucrose-[U-$^{14}$C] by excised apices was restricted by anoxia mainly to conversion to ethanol, $CO_2$, alanine and glycolytic intermediates. Measurements of metabolites over a period of 240 min after transfer of excised apices to nitrogen showed a marked and continual accumulation of ethanol, a smaller continual accumulation of alanine, a small initial rise in lactate and no detectable accumulation of malate or pyruvate. The rates of $CO_2$ production, of accumulation of ethanol and alanine, and of the labelling of these compounds by sucrose-[$^{14}$C] declined markedly during the first 240 min of anaerobiosis. The conclusion is that under anaerobic conditions carbohydrate metabolism in the pea root apex is largely restricted to alcoholic fermentation, and, to a lesser degree, to alanine production.

## INTRODUCTION

Water-logging of soils can subject plant roots to considerable periods of anaerobiosis [1, 2]. The detailed response of carbohydrate oxidation, the prime source of energy in the root, to anaerobiosis is known for the roots of extremely few species. The available data indicate that alcoholic fermentation is widespread and often accounts for much of the carbohydrate consumed in anaerobiosis. However, it is clear that this production of ethanol is generally accompanied by other forms of fermentation. Lactate is known to be formed and in some instances to be the major product of fermentation [3]. The existence of other pathways of fermentation is suggested by reports that in certain species anaerobiosis may lead of the accumulation of a number of compounds, including alanine [4], γ-aminobutyrate [5], malate [6] and glycerol [1]. There have been suggestions that variation in the products of fermentation is important in respect of the ability of plant tissues to withstand anaerobiosis. Crawford [7] has proposed that tolerance of anaerobiosis is associated with an ability to produce compounds other than ethanol, and has suggested malate and other organic acids as important alternatives. Davies [8] has proposed that, at the onset of anaerobiosis, acids such as lactate and malate accumulate as the main products of fermentation. This was held to cause a fall in cytoplasmic pH which restricted further production of acid and led to the formation of ethanol as the main product of fermentation. The switch from acids to ethanol was attributed to differences in the pH optima of pyruvate decarboxylase and lactate dehydrogenase, and the sensitivity of the latter to ATP at low pH. Assessment of the above hypotheses requires detailed knowledge of the effects of anaerobiosis on carbohydrate breakdown. The aim of the work reported in this paper was to provide such knowledge for roots of a plant known to be particularly intolerant of anaerobic soils, *Pisum sativum* [9].

We studied the apical 6 mm of the roots of aerobically grown 5-day-old seedlings. The high rate of respiration [10], and the relative lack of air spaces [11], indicate that this is the region of the pea root which is most likely to become anaerobic in the field. We used three experimental approaches. First, we investigated the capacities of the apices to catalyse different fermentations. We did this by estimating the maximum catalytic activities of alcohol dehydrogenase (EC 1.1.1.1), lactate dehydrogenase (EC 1.1.1.27), and phosphoenolpyruvate carboxylase (EC 4.1.1.31). The latter was chosen as an indicator of the capacity to form malate and related compounds. We also measured NADP-specific malic enzyme (EC 1.1.1.40) because Crawford [1] has suggested that variation in its activity may affect which compounds accumulate during anaerobiosis. Our second approach was to determine the effects of anaerobiosis on the distribution of label from sucrose-[U-$^{14}$C]. The apex of pea roots receives its carbohydrate as sucrose [12]. Thus the above experiments were designed to reveal the general effects of anaerobiosis on carbohydrate metabolism, and to indicate the full range of products of sugar fermentation. Finally, we measured the extent to which compounds, indicated by the labelling experiments to be products of fermentation, accumulated under anaerobic conditions. We did this to estimate the relative activities of the pathways of fermentation. We paid particular attention to the difficulties of measuring enzymes and substrates in plant tissues [13].

## RESULTS

### *Enzyme activities*

We investigated the reliability of our assays of enzyme activities in the following ways. Firstly, we optimized the concentration of each component, and the pH, of the assay mixtures for each enzyme. Addition of malate dehydrogenase and bicarbonate to the assay mixture for phosphoenolpyruvate carboxylase did not increase the observed activity as the amounts of malate dehydrogenase in the extract, and of bicarbonate in the solutions, were sufficient to give maximum rates. Substitution of $Mg^{2+}$ for $Mn^{2+}$ in the assay for malic enzyme gave lower activities over the range pH 6.6–8.5. Secondly, we showed that the activity for each enzyme was dependent upon the presence of the appropriate substrate, was linear for at least 4 min, and was directly proportional to the amount of extract assayed. We demonstrated that 85% of the activity recorded for phosphoenolpyruvate carboxylase was dependent upon added $Mg^{2+}$. By using $CO_2$-free solutions, we showed that 80% of the activity measured for this enzyme was dependent upon the presence of bicarbonate. The small amount of activity observed in the absence of bicarbonate was probably due to the high affinity of the enzyme for its substrate and an inability to keep the reaction mixture entirely free of bicarbonate.

In order to discover if enzyme activity was being lost during the preparation of the extracts, we carried out two checks. In the first we determined whether the extracts affected the activities of samples of pure enzymes. For each test we prepared duplicate samples of apices, homogenized one in extraction buffer and the other in extraction buffer which contained a measured amount of purified enzyme. The latter was comparable to the activity found in the sample of roots. Comparison of the activities recovered from the duplicate samples was used to estimate the percentage of the purified enzyme which survived homogenization and extraction. Values of 85, 100 and 115% were obtained for alcohol dehydrogenase, lactate dehydrogenase and phosphoenolpyruvate carboxylase, respectively. Pure malic enzyme was not available. For the second check we compared enzyme activities of a sample of pea root apices, a sample of the apical 2 cm of the roots of *Ranunculus sceleratus*, and a sample which was a mixture of pea and *Ranunculus* roots. The actual activity found for the mixed sample was expressed as a percentage of the value predicted from the measurements made on the separate components of the mixture. Significant disagreement between the observed and predicted values would indicate inhibition or activation of the enzyme by component(s) of one of the extracts. Values of 93, 92, 141 and 86% were obtained for alcohol dehydrogenase, lactate dehydrogenase, phosphoenolpyruvate carboxylase and malic enzyme, respectively. The value for the carboxylase suggests some activation of the enzyme by the extract of one of the tissues. Apart from this, the results of the above checks indicate that there was no substantial loss or activation of the enzymes during extraction. From this evidence, and our optimization of the assays, we suggest that our measurements reflect the maximum catalytic activities of the pea root apices.

Our estimates of the enzyme activities are presented in Table 1, together with comparable data for some glycolytic enzymes known to mediate the respiration of the roots [10]. The activities of alcohol dehydrogenase, lactate dehydrogenase, phosphoenolpyruvate carboxylase and malic enzyme are roughly comparable to that of phosphofructokinase, an enzyme which catalyses a step likely to limit glycolysis. However, the activity of alcohol dehydrogenase was greater than that of any of the other enzymes which we measured.

### *Metabolism of sucrose-*[$^{14}$C]

We determined the effects of anaerobiosis on the metabolism of sucrose-[U-$^{14}$C] to $CO_2$, the water-insoluble material, and the acidic and basic components of the water-soluble material of the apices. The total label recovered in the above fractions is summed to give what is referred to as $^{14}$C metabolized. We found that anoxia caused extensive leakage of labelled metabolites from the apices. In 60 min incubations, 35% of the label recovered in the basic components and 87% of that in the acidic components was found in the suspending medium plus the solution used to rinse the roots before killing. The corresponding figures for incubations of 330 min were 91 and 95%. Even when the apices were incubated for 330 min under aerobic conditions, 9 and 35%, respectively, of the label in the basic and acidic components were recovered in the medium plus the rinsings. Because of this leakage, in all subsequent experiments we combined the tissue extract and the suspending medium, and then determined the distribution of the $^{14}$C.

The effects of anaerobiosis on sucrose-[$^{14}$C] metabolism are exemplified by the data in Table 2; comparable data were obtained with separately grown batches of roots. In these experiments, and those reported in Fig. 1, very little label was lost during the analyses. The sum of

Table 1. Activities of glycolytic and related enzymes in pea root apices

| Enzyme | Activity (nkat/mg protein)* |
|---|---|
| Alcohol dehydrogenase | 2.13 ± 0.32 (6) |
| Lactate dehydrogenase | 0.67 ± 0.08 (6) |
| Phosphoenolpyruvate carboxylase | 1.48 ± 0.03 (6) |
| NADP-specific malic enzyme | 0.75 ± 0.05 (6) |
| Phosphofructokinase† | 1.58 ± 0.05 (7) |
| Fructose 1,6-bisphosphate aldolase† | 5.18 ± 0.38 (7) |
| NAD-specific glyceraldehydephosphate dehydrogenase† | 4.08 ± 0.88 (5) |

* Values are means ± S.E. of assays made on the number of extracts shown in parentheses.

† These values, from Fowler and ap Rees [10], refer to the apical 5 mm of the root; all other values refer to the apical 6 mm.

Table 2. Effects of anaerobiosis on the metabolism of sucrose-[U-$^{14}$C] by the apical 6 mm of pea roots

| | Aerobic roots | Anaerobic roots |
|---|---|---|
| $^{14}$C metabolized as percentage of that supplied | 47.0 | 6.7 |
| Percentage of metabolized $^{14}$C recovered as: | | |
| $CO_2$ | 36.5 | 22.5 |
| Water-insoluble substances | 38.0 | 2.0 |
| Water-soluble substances | 25.5 | 75.5 |
| Acidic components | 11.0 | 33.5 |
| Glycolytic intermediates | 8.5 | 29.3 |
| Lactate | 1.4 | 1.0 |
| Malate | 0.4 | 0.4 |
| Basic components | 14.5 | 42.0 |
| Alanine | 5.5 | 31.0 |
| Aspartate | 4.0 | 2.5 |
| Glutamate | 2.5 | 2.5 |

Samples (0.33 g fr. wt) were incubated in 0.5 μCi sucrose-[U-$^{14}$C] at 0.1 mM in 0.02 M $KH_2PO_4$, pH 5.2, for 330 min, and were then killed and extracted with boiling 80% aq. ethanol. The $^{14}$C recovered in the acidic and basic components of the water-soluble substances, in the water-insoluble substances, and in $CO_2$ was summed to give $^{14}$C metabolized.

the label recovered in the acidic, basic and neutral components amounted to at least 90% of that present in the water-soluble substances. The label recovered in the different constituents of the basic and acidic components after paper chromatography accounted for at least 87% of that applied to the chromatograms. The following points are made from Table 2. First, anoxia drastically reduced the amount of sucrose-[$^{14}$C] metabolized. Second, labelling of the insoluble material, extensive under aerobic conditions, was almost completely stopped by anoxia. Third, under both aerobic and anaerobic conditions, most of the $^{14}$C in the acidic components was recovered from those regions of the chromatograms occupied by hexose phosphates, triose phosphates, 3-phosphoglycerate, phosphoenolpyruvate and pyruvate. As these compounds were not completely separated by the solvents used, the label is collectively assigned to glycolytic intermediates. Very little label was recovered in lactate, malate or related organic acids under either aerobic or anaerobic conditions. Finally, anaerobiosis led to a marked increase in the proportion of metabolized label found in the basic fraction; this increase was due almost entirely to a massive rise in the proportion recovered as alanine-[$^{14}$C].

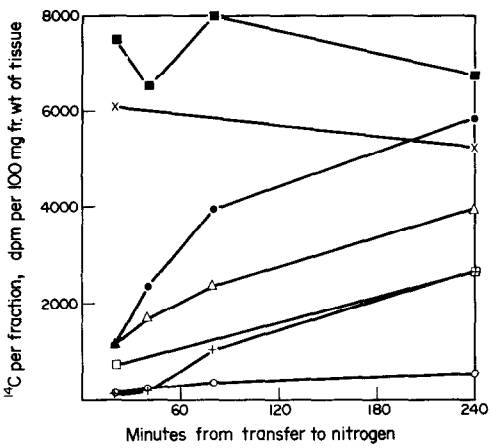


Fig. 1. Metabolism of sucrose-[U-$^{14}$C] by pea root apices under anaerobic conditions. Samples (0.7 g fr. wt) were suspended in 3.5 ml $KH_2PO_4$, pH 5.2 and made anaerobic. Then 0.5 μCi sucrose-[U-$^{14}$C], at 0.6 mM in 0.5 ml 0.02 M $KH_2PO_4$, pH 5.2, was added to each sample. At the end of the incubation, each sample and its suspending medium was added to liquid nitrogen and then killed and extracted with 1.41 M $HClO_4$. The extracts were fractionated to give: ■———■, acidic components of water-soluble substances; ×———×, glycolytic intermediates; ●———●, ethanol; △———△, basic components of water-soluble substances; □———□, alanine; +———+, $CO_2$; ○———○, water-insoluble substances.

In order to discover if the anaerobic metabolism of sucrose varied according to the period of anaerobiosis, we determined the time-course of the labelling of the principal compounds formed from sucrose-[$^{14}$C] (Fig. 1). In these experiments, metabolism was stopped with liquid nitrogen and the tissue was killed with cold $HClO_4$. This permitted us to determine the labelling of ethanol. The following points are demonstrated by Fig. 1. Ethanol and $CO_2$ were heavily and continuously labelled, roughly in the ratio of 2:1. There was no evidence of any lag in the labelling of ethanol. The labelling of alanine was always significant and showed a time-course similar to that of ethanol. The amount of label recovered in glycolytic intermediates rose rapidly and then remained more or less constant. Finally, the rates at which all the other fractions or compounds were labelled declined markedly during the 240 min of anaerobiosis despite the fact there was still plenty of sucrose-[$^{14}$C] present.

*Measurements of metabolites*

We measured the extent to which ethanol, alanine, pyruvate, lactate and malate accumulated during 240 min anaerobiosis. Ideally such measurements should be made on freeze-clamped tissue and be accompanied by evidence that no significant losses occurred during killing and extraction [13]. Such evidence is probably best provided by adding measured amounts of the metabolites to a sample of tissue immediately after freeze-clamping and then showing that the exogenous compounds are recovered in full after killing and extracting the tissue. This was not practicable with samples of anaerobically

incubated roots because freeze-clamping involved exposure of the samples to air and the consequent risk of changes in the amounts of the metabolites as aerobic metabolism was resumed. We avoided this risk by stopping metabolism by tipping the samples straight into liquid nitrogen.

The following is evidence that the above procedure was satisfactory. We freeze-clamped duplicate samples of freshly excised apices from aerobic roots. To one sample we added, immediately after freeze-clamping, the following (in μmol): ethanol 40, alanine 5, pyruvate 0.15, lactate 2 and malate 3. Both samples were then killed, extracted and analysed. The recoveries of the added compounds were calculated from the differences between the amounts obtained from the two samples. This type of experiment was carried out with three pairs of samples, each pair being from a separately grown batch of peas. The average recoveries for each of the above compounds were estimated: all the values lay between 87 and 108% of the amounts added. We conclude that there were no appreciable losses of the compounds when the roots were freeze-clamped. Next we compared the amounts of the above compounds which we could detect in a freeze-clamped sample with the amounts found in a replicate sample which had been tipped into liquid nitrogen. We made such comparisons between three pairs of samples of aerobic roots, and between three pairs of samples of roots which had been kept anaerobic for 240 min. For each pair of samples, the estimate for the frozen sample was expressed as a percentage of that of the freeze-clamped sample. For aerobic and anaerobic roots, the mean values which we obtained for ethanol, alanine, pyruvate, lactate and malate were in the range 96–116%. This shows that freezing the apices in liquid nitrogen was as effective as freeze-clamping in stopping metabolism. As the recoveries of the compounds from the freeze-clamped samples have been shown to be satisfactory, we conclude that the estimates from the frozen samples are reliable.

The effects of anaerobiosis on the amounts of the different compounds in the excised apices are shown in Table 3. The amounts present in the apices before transfer to anaerobic conditions varied with the batch of seedlings. However, the changes in the amounts during anaerobiosis showed less variation and the data are given as such changes. There were always appreicable quantities of ethanol in aerobically grown roots even when we grew seedlings with their roots in a moist atmosphere but not in direct contact with water. The following additional points are made. Of all the compounds measured, ethanol showed by far the largest increase during anaerobiosis. There were appreciable increases in the amounts of alanine, but no changes in the amounts of pyruvate were detected and the amounts of malate fell appreciably. Ethanol and alanine accumulated throughout the period of anaerobiosis but the rate of accumulation declined considerably with time. The amount of lactate present rose during the first 20 min of anaerobiosis but changed little thereafter.

The other product of fermentation which we measured was $CO_2$ (Fig. 2). The rate declined almost continuously throughout the period of anaerobiosis. This decline was not arrested by carrying out the incubation in nitrogen

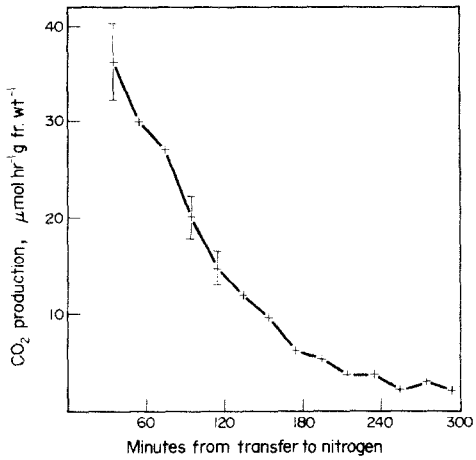


Fig. 2. $CO_2$ production by pea root apices after transfer to anaerobic conditions. Samples were gassed with oxygen-free nitrogen for 15 min and then equilibrated for a further 15 min. Transfer to nitrogen is measured from the start of the equilibration period. Each point is the mean of estimates from 3 samples; where the S.E. is greater than 10% of the mean, the former is indicated by bars.

Table 3. Changes in contents of glycolytic and related metabolites during anaerobiosis of excised apices of pea roots

| | Ethanol | Alanine | Pyruvate | Lactate | Malate |
|---|---|---|---|---|---|
| Amount present before transfer to anaerobic conditions (μmol/g fr. wt) | 38.0 ± 6.9 | 5.36 ± 0.60 | 0.15 ± 0.03 | 1.70 ± 0.32 | 2.70 ± 0.17 |
| Change in amount (μmol/g fr. wt) after transfer to anaerobic conditions for: | | | | | |
| 20 min | 8.9 ± 2.5 | 2.33 ± 0.33 | 0.03 ± 0.02 | 1.18 ± 0.32 | |
| 40 min | 17.8 ± 2.3 | 3.85 ± 0.51 | 0.05 ± 0.02 | 1.39 ± 0.32 | −0.76 ± 0.10 (4) |
| 60 min | 22.3 ± 2.4 | 3.94 ± 0.59 | 0.02 ± 0.02 | 1.63 ± 0.30 | |
| 80 min | 24.8 ± 4.4 | 3.94 ± 0.59 | 0.02 ± 0.03 | 1.73 ± 0.36 | |
| 240 min | 37.0 ± 3.1 | 4.99 ± 0.37 | −0.01 ± 0.17 | 1.46 ± 0.28 | −2.29 ± 0.17 (5) |

In each experiment 6 samples were prepared, one was analysed at once and the other five were made anaerobic. The latter were analysed after different periods of anaerobiosis as indicated. Values are means ± S.E. of estimates from 6 experiments, each with a different batch of plants, except where the number of experiments is given in parentheses. Changes in content are increases unless shown otherwise.

in the presence of either 0.15 or 4 mM sucrose in 0.02 M $KH_2PO_4$ (pH 5.2). This indication that the decline was not due to lack of substrate was supported by comparing the gas exchange of excised spices which had been incubated aerobically for 200 min with that of excised apices which had been kept anaerobic for 200 min and then returned to air. The $CO_2$ production and the oxygen uptake of the latter samples were found to be similar to those of the former.

## DISCUSSION

The measurements of enzyme activities indicate that pea root apices have the capacity to make ethanol, lactate and malate, either singly or in combination, the major products of fermentation of carbohydrate. However, the distribution of label from sucrose-[$^{14}$C] and the measurements of the accumulation of the products of fermentation show that alcoholic fermentation is the major route of carbohydrate breakdown under anaerobic conditions, and that only a very small fraction of fermentation results in the accumulation of organic acids. Ethanol and $CO_2$ were the amjor or labelled products of sucrose-[$^{14}$C] fermentation and they accumulated in greater amounts than any other product. The labelling data indicate that fermentation of carbohydrate produced ethanol and $CO_2$ in the ratio of 2:1. Alanine was the only other compound, formed from sucrose, which accumulated in substantial amounts. This is shown by both the labelling data, and the measurements in Table 3. As stated in the Introduction, the accumulation of alanine under anaerobic conditions has been demonstrated for other plants. The pathway of alanine synthesis in anaerobic roots, and the significance of its formation, have not been established. Alanine is presumably formed by transamination of pyruvate formed in glycolysis. It is probable that such transamination occurs at the expense of some amino acid(s) which yields an organic acid, the metabolism of which directly or indirectly oxidizes NADH.

Although the data in Tables 2 and 3 show that fermentation of carbohydrate did not lead to any appreciable accumulation of organic acids, lactate did increase in the first 20 min of anaerobiosis. This increase was small in absolute terms and in relation to the simultaneous increase in ethanol, and was largely confined to the first 20 min. This initial production of lactate is consistent with Davies' hypothesis that lactate accumulates only in the early stages of anaerobiosis. However, we found no evidence that cessation of lactate accumulation was accompanied by a switch to ethanol production. Our results do not rule out this possibility. This is because the apices contained appreciable amounts of lactate and ethanol even before they were transferred to nitrogen. This appears to be a natural phenomenon as it occurred even when the roots were grown with excellent aeration. Thus even in very well aerated soils there will be some fermentation in the pea root apex. Consequently a major switch from lactate to ethanol production could have occurred during the natural growth and development of the root apex and have been more or less complete before we transferred the apices to nitrogen.

Regardless of whether Davies' hypothesis is applicable to pea roots, our results strongly indicate that under anaerobic conditions carbohydrate metabolism in the root apex is largely limited to alcoholic fermentation and the production of alanine. Our labelling experiments show that biosynthesis from sugars and their immediate derivatives is almost completely stopped. It is also clear that the rate, as well as the extent, of carbohydrate metabolism is severely reduced by prolonged anaerobiosis. The rates at which the products of fermentation accumulated and became labelled all declined very markedly during the first 200 min of anaerobiosis. There is evidence that anaerobiosis in plant tissues is often accompanied by a stimulation of the rate of carbohydrate oxidation [14]. If such a stimulation occurs in the excised apices of pea roots, then it is remarkably short-lived.

There is insufficient data available for us to decide whether the response of pea root apices to anoxia is typical of the roots of plants which do not tolerate a lack of oxygen. A particular difficulty in comparing our results with published work is posed by our demonstration that anoxia causes extensive leakage of metabolites from pea root apices. This makes it difficult to interpret analyses of the products of fermentation and of the distribution of $^{14}$C under anaerobic conditions unless the possibility of leakage has been taken into account.

## EXPERIMENTAL

*Materials.* Sucrose-[U-$^{14}$C] was from The Radiochemical Centre, Amersham, and enzymes and co-factors from Boehringer except for fructose-6-phosphate, acetylpyridine adenine dinucleotide and aspartate aminotransferase which were from Sigma. Seeds of *Pisum sativum* L. cv Kelvedon Wonder were soaked in 10% (v/v) HOCl for 10 min, washed in running $H_2O$ for 24 hr, placed between paper towels dampened with 0.2 mM $CaCl_2$ and incubated in plastic boxes in the dark at 25° for 48 hr. Then seedlings with well developed roots were selected, supported with their roots projecting through plastic netting floating on continuously and very vigorously aerated 0.2 mM $CaCl_2$, and grown for a further 48 hr in the dark at 25°. The apical 6 mm of the roots of these 5-day-old seedlings were used as experimental material; apices were excised immediately before use. Seeds of *Ranunculus sceleratus* L. were germinated in wet sand, and the seedlings were grown for 30 days in aerated solns which contained 2 mM $KNO_3$, 1 mM $KH_2PO_4$, 0.5 mM $MgCl_2$, 2.0 mM $CaCl_2$, 0.085 mM ferric-EDTA complex, and trace elements. The apical 2 cm of the roots were used as experimental material.

*Assay of enzymes.* Samples (fr. wt 0.5 g) of root apices were homogenized, with a pestle and mortar and then with an all-glass homogenizer, in 6 vols. 50 mM glycylglycine buffer (pH 7.4) which contained 10 mM 2-mercaptoethanol. Examination of the homogenates with a microscope revealed very few unbroken cells. Each homogenate was centrifuged at 100000 *g* for 30 min and the supernatant was assayed at once. Extracts were prepared at 4° and kept at 2° until assayed at 25° in reaction mixtures of 3.0 ml as indicated in the following references. These mixtures contained: alcohol dehydrogenase [15], 100 mM ethanol, 1.26 mM NAD in 46.7 mM glycylglycine, pH 8.6; lactate dehydrogenase [16], 1.5 mM pyruvate, 0.24 mM NADH in 45 mM MES buffer, pH 6.7; phosphoenolpyruvate carboxylase [17], 1.43 mM phosphoenolpyruvate, 1.64 mM $MgCl_2$, 0.12 mM NADH in 41.7 mM glycylglycine, pH 8.6; NADP-specific malic enzyme [18], 2.14 mM malate, 0.42 mM NADP, 0.84 mM $MnCl_2$ in 45 mM MOPS buffer, pH 7.6. Protein in the extracts was determined as in ref. [19].

*Metabolism of sucrose-*[$^{14}$C]. For the expts in which the tissue was killed with EtOH, duplicate samples of apices (0.3 g fr. wt)

were prepared and each was suspended in 2.0 ml 0.02 M $KH_2PO_4$ (pH 5.2) in a Warburg flask. One flask contained air, and the other was gassed with $O_2$-free $N_2$ for 15 min. The taps were then closed and 0.5 μCi sucrose-[U-$^{14}$C] at 0.6 mM in 0.5 ml 0.02 M $KH_2PO_4$ (pH 5.2) was tipped from the side arm onto each sample; $^{14}CO_2$ was collected in alkali in the centre well. At the end of the incubation, the alkali was quickly removed and the tissue and suspending medium were poured into boiling 80% aq. EtOH. The tissue was extracted successively in boiling 80% aq. EtOH and boiling 60% aq. EtOH. The residue is referred to as water-insoluble substances and its $^{14}$C content was measured as in ref. [12]. The extracts were combined, reduced to 3–4 ml at 28° to give the water-soluble substances which were divided into their acidic, basic, and neutral components by ion-exchange chromatography as in ref. [19]. Further analysis was by PC on Whatman No. 1 paper with the following solvents: acidic components, *n*-BuOH–HOAc–$H_2O$ (12:5:5), water-saturated PrOH–$HCO_2H$–cineole (5:2:5) upper phase; basic components, *n*-BuOH–HOAc–$H_2O$ (12:5:5), PhOH–$H_2O$ (4:1). Compounds were located by using authentic substances as markers. Acidic compounds were revealed with bromocresol green, and amino acids with ninhydrin. Labelled compounds were eluted from the paper before being counted.

When leakage from the apices was studied samples were prepared, incubated and analysed as above except that at the end of the incubation each sample was removed from the medium, rinsed with 3 × 2.0 ml portions of 0.02 M $KH_2PO_4$ (pH 5.2), and then put into boiling 80% aq. EtOH. The rinsings and the incubation medium were combined and analysed separately from the tissue extracts.

In the expts in which labelled apices were killed with cold $HClO_4$, 4 replicate samples, each of 0.7 g fr. wt, were prepared. Each sample was placed in 3.5 ml 0.02 M $KH_2PO_4$ (pH 5.2) in a 125 ml Erlenmeyer flask fitted with a centre well which contained alkali. Each flask was quickly gassed by passing 600 ml $O_2$-free $N_2$ through it in 2 min. The flasks were then sealed and 0.5 μCi sucrose-[U-$^{14}$C] at 0.6 mM in 0.5 ml 0.02 M $KH_2PO_4$ (pH 5.2) was added to each sample by injection through a self-sealing bung. At the end of the incubation, at 25°, the alkali was removed from the centre well, and the tissue and medium were rapidly and quantitatively transferred to liquid $N_2$. Before the latter had evapd, sufficient 2.82 M $HClO_4$ at 1° was added to each sample to give a concn of 1.41 M in the final extract. The samples were left at −10° for 18 hr, and were then extracted as described in ref. [20] except that treatment with charcoal was omitted. The extract was fractionated by ion-exchange and paper chromatography, and the $^{14}$C in the insoluble substances was determined as described above. EtOH was separated from the extract by distillation at pH 9.

*Measurement of metabolites.* Samples of tissue (0.7–1.3 g fr. wt) were prepared and either analysed at once or after incubation under anaerobic conditions. The latter was achieved by placing each sample in 4.0 ml 0.02 M $KH_2PO_4$ (pH 5.2) in a 125 ml Erlenmeyer flask which was gassed with $O_2$-free $N_2$ and incubated as described for the expts with sucrose-[$^{14}$C]. Freeze-clamping and killing of the freeze-clamped tissue was carried out as in ref. [20]. When metabolism was stopped with liquid $N_2$, the sample and the medium were tipped straight into the liquid $N_2$, and then 2.82 M $HClO_4$ was added as described for the samples labelled by sucrose-[$^{14}$C]. The transfer from anaerobic conditions to liquid $N_2$ took 3 sec. Freeze-clamped and frozen samples were left at −10° for 18 hr and were then extracted with 1.41 M $HClO_4$ as described above. Metabolites in the extracts were assayed spectrophotometrically by the methods described in the following refs.: EtOH [21], alanine [22], pyruvate [23], lactate [24], malate [25]. Gas exchange was measured manometrically by Warburg's direct method with samples of 0.3–0.5 g fr. wt suspended in 2.0 ml 0.02 M $KH_2PO_4$ (pH 5.2) at 25°. Samples were made anaerobic by gassing the flasks with $O_2$-free $N_2$ for 15 min. $^{14}$C was measured by liquid scintillation spectrometry as in ref. [26].

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## REFERENCES

1. Crawford, R. M. M. (1972) *Trans. Proc. Bot. Soc. Edinburgh* **41**, 309.
2. Harborne, J. B. (1977) *Introduction to Ecological Biochemistry*, pp. 9–10. Academic Press, New York.
3. Thomas, M., Ranson, S. L. and Richardson, J. A. (1973) *Plant Physiology*, 5th edn, pp. 609–620. Longmans, London.
4. Effer, W. R. and Ranson, S. L. (1967) *Plant Physiol.* **42**, 1042.
5. Streeter, J. G. and Thompson, J. F. (1972) *Plant Physiol.* **49**, 572.
6. Crawford, R. M. M. (1967) *J. Exp. Botany* **18**, 458.
7. Crawford, R. M. M. (1969) *Ber. Dtsch. Bot. Ges.* **82**, 11.
8. Davies, D. D. (1973) *Symp. Soc. Exp. Biol.* **27**, 513.
9. Cannell, R. Q., Gales, K. and Suhail, B. A. (1976) *Annual Report Letcombe Laboratory Wantage*, pp. 67–69. HMSO, London.
10. Fowler, M. W. and ap Rees, T. (1970) *Biochim. Biophys. Acta* **201**, 33.
11. Popham, R. A. (1955) *Am. J. Botany* **42**, 529.
12. Dick, P. S. and ap Rees, T. (1976) *Phytochemistry* **15**, 255.
13. ap Rees, T. (1974) *MTP Int. Rev. Sci. Biochem.* **11**, 89.
14. Beevers, H. (1960) *Respiratory Metabolism in Plants*, pp. 150–156. Row Peterson, Evanston.
15. Cossins, E. A., Kopala, L. C., Blawacky, B. and Spronk, A. M. (1968) *Phytochemistry* **7**, 1125.
16. Davies, D. D. and Davies, S. (1972) *Biochem. J.* **129**, 831.
17. Wong, K. F. and Davies, D. D. (1973) *Biochem. J.* **131**, 451.
18. Johnson, H. S. and Hatch, M. D. (1970) *Biochem. J.* **119**, 273.
19. Stitt, M. and ap Rees, T. (1978) *Phytochemistry* **17**, 1251.
20. ap Rees, T., Fuller, W. A. and Wright, B. W. (1977) *Biochim. Biophys. Acta* **461**, 274.
21. Witt, I. (1974) *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.) 2nd edn, Vol. 3, pp. 1502–1505. Academic Press, New York.
22. Williamson, D. H. (1974) *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.) 2nd edn, Vol. 4, pp. 1679–1682. Academic Press, New York.
23. Bücher, T., Czok, R., Lamprecht, W. and Latzko, E. (1963) *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.) 1st edn, pp. 253–259. Academic Press, New York.
24. Gutman, I. and Wahlesfeld, A. W. (1974) *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.) 2nd edn, Vol. 3, pp. 1464–1468. Academic Press, New York.
25. Möllering, H. (1974) *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.) 2nd edn, Vol. 3, pp. 1589–1593. Academic Press, New York.
26. Wong, W. J. L. and ap Rees, T. (1971) *Biochim. Biophys. Acta* **252**, 296.