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EFFECTS OF LACK OF OXYGEN ON THE METABOLISM OF SHOOTS OF *TYPHA ANGUSTIFOLIA*

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Key Word Index—*Typha angustifolia*; Typhaceae; lesser reedmace; shoot; hypoxia; [^{14}C]sucrose metabolism; protein synthesis.

Abstract—Excised young shoots of *Typha angustifolia* were incubated with [U-^{14}C]sucrose in air, nitrogen and mixtures containing 14, 11, 8, 6, 4, 2 and 1% oxygen. Total ^{14}C metabolized was not significantly affected by oxygen concentration. The percentage of metabolized ^{14}C recovered in the fractions that contained the major macromolecules was not reduced until the oxygen concentration reached 1% and was appreciable even in anoxia. Pulse and chase experiments with [^{14}C]valine confirmed appreciable protein synthesis in anoxia and indicated that 90% of the anoxically synthesized protein was in the cell-wall and the membrane fractions of the shoot. This behaviour of *Typha* shoots, which had no living connexion with the atmosphere, is contrasted with that of a wetland plant with well developed aerenchyma.

INTRODUCTION

When excised roots of the flood tolerant *Glyceria maxima* and the flood intolerant *Pisum sativum* were incubated in [^{14}C]sucrose, lowering the oxygen concentration greatly reduced the percentage of metabolized ^{14}C that was recovered in the root fractions that contained the major macromolecules; polysaccharide, protein, nucleic acid and lipid. Anoxia reduced this labelling to the point at which it could barely be detected. Both the degree of reduction in labelling of the macromolecules and the oxygen concentration at which this reduction became apparent, 4–6%, were very similar in both types of root [1]. Thus, although *Glyceria maxima* grows well in soils that lack oxygen [2], its roots, when made anoxic, are largely incapable of converting sucrose, their normal source of carbon, to the major macromolecules formed during growth. Our observations are consistent with those of Barclay and Crawford [3] who showed that *Glyceria maxima* was amongst the majority of the wetland plants that they studied in that it made no detectable growth when the whole plant was made anoxic. However, a small number of plants have been shown to make at least some growth in anoxia [3]. The aim of the work described in the present paper was to discover if the young shoots of one of these plants, *Typha angustifolia*, responded to oxygen deficiency differently from the roots of *Glyceria maxima* in respect of [^{14}C]sucrose metabolism. Our experimental approach was similar to that used earlier [1] in that we determined the effects of anoxia and a range of oxygen concentrations on the detailed distribution of label after supplying [U-^{14}C]sucrose to excised shoots of *Typha angustifolia*.

RESULTS AND DISCUSSION

Our experimental material was young shoots, 1–2.3 g fr. wt, freshly excised from rhizomes growing naturally in mud covered with at least 20 cm of water. We stress that the excised shoots included their basal meristem and were otherwise undamaged. In the plants that we sampled the only connexions between the rhizomes and the atmosphere were dead sodden leaves of previous years' growth. The redox potential at rhizome depth ranged from +232 to +249. The oxygen concentration in water taken from the surface of the mud was 6%. Thus our experimental material came from naturally hypoxic sites.

We suspended the shoots in buffer in flasks that we gassed with appropriate mixtures of oxygen and nitrogen for 45 min. Then [U-^{14}C]sucrose was added and incubation was continued, with gassing, for a further 5 hr when it was stopped by the addition of perchloric acid. The tissue was extracted and the water extract and the suspending medium were added together to give the water-soluble substances, which were then analysed by ion-exchange chromatography. The residue was next extracted with ethanol to give the water-insoluble ethanol-soluble substances, and the water- and ethanol-insoluble substances. The latter were incubated with amyloglucosidase and α-amylase to release ^{14}C present in starch, and then with Pronase to obtain ^{14}C present in protein. Most of the label in the water- and ethanol-insoluble fraction that resisted solubilization by the above enzymes is likely to have been in structural polysaccharides.

The relative rarity of *Typha angustifolia* made it difficult to obtain identical shoots that contained the same number of actively metabolizing cells. To take into account sample variation, and the possibility that changes in oxygen concentration might affect the amount of ^{14}C taken up and metabolized, for each sample we have expressed the ^{14}C recovered in the tissue fractions as a percentage of the

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sum of ^{14}C recovered in the water-insoluble ethanol-soluble substances, the water- and ethanol-insoluble substances, the basic and acidic components of the water-soluble substances and as $^{14}CO_2$. For convenience we refer to this sum as the ^{14}C metabolized (Table 1). There was little loss of label during the analyses. The total ^{14}C recovered in $^{14}CO_2$, water-soluble ethanol-insoluble substances, water- and ethanol-insoluble substances, and water-soluble substances, when summed and expressed as a percentage of the ^{14}C supplied to each of the samples described in Table 1 was 95 ± 2 (mean $\pm$ s.e. of all nine samples). In no sample was more than 3% of the ^{14}C in the water-soluble substances lost during the ion-exchange chromatography.

At each concentration of oxygen the shoots of *Typha angustifolia* metabolized appreciable amounts of the [^{14}C]sucrose (Table 1). The amounts metabolized per g fr. wt or per shoot did not differ significantly with the oxygen concentration. This contrasts strikingly with the behaviour of roots of *Glyceria maxima* and pea where hypoxia led to a marked decline in the amount of [^{14}C]sucrose metabolized. For example, the [^{14}C]sucrose metabolized by *Glyceria* roots in nitrogen was one third of that in air [1]. The second point we stress from Table 1 is that, although hypoxia and anoxia reduced the labelling of the insoluble fractions, which contained the major macromolecules made during growth, the oxygen concentration at which this reduction became obvious, 1%, was appreciably lower for *Typha angustifolia* shoots than it was for roots of *Glyceria maxima* and pea, 4% [1]. Finally, and most importantly, we point out that, although incorporation into macromolecules in the *Typha* shoots was reduced by hypoxia, the percentage reduction was very much less than in the roots of *Glyceria* and pea. Even in anoxia there was appreciable labelling of the insoluble fractions of *Typha* shoots. For the latter the percentage of metabolized ^{14}C recovered in substances insoluble in water was 14, 13 and 19 for shoots in nitrogen, 1% and 2% oxygen, respectively (Table 1). For *Glyceria* roots, the corresponding figures were 3, 3 and 8% [1]. Our analysis of the distribution of the ^{14}C recovered in the water-insoluble fractions of *Typha* shoots strongly suggests that in hypoxia and anoxia there was appreciable synthesis of a wide range of macromolecules: starch, lipid (water-insoluble, ethanol-soluble), cell-wall polysaccharides and protein.

We confirmed the ability of shoots of *Typha angustifolia* to convert sucrose to macromolecules in anoxia by supplying [U-^{14}C]sucrose, anoxically, to shoots that were still attached to their parent rhizomes. In these experiments we took a small piece of rhizome bearing a single shoot 4–8 cm long, and kept it in 95% nitrogen–5% carbon dioxide for seven (first experiment) or three (second experiment) days before supplying the shoot still attached to the rhizome in the above gas mixture, with [U-^{14}C]sucrose for 6 hr. Incorporation of ^{14}C into water-insoluble material was 5 and 24% of the metabolized label in the first and second experiments, respectively.

The fact that we could detect appreciable labelling of protein by [^{14}C]sucrose in anoxia with shoots of *Typha angustifolia* (Table 1) but not with roots of *Glyceria maxima* [1] led us to investigate the intracellular distribution of the anoxically formed protein in the shoots. We did this by following the incorporation of [U-^{14}C]valine into protein in three major sub-cellular fractions of the shoot in pulse and chase experiments (Table 2). Excised shoots were subjected to 45 min anoxia and then, still in anoxia, supplied with [U-^{14}C]valine for 6–8 hr (pulse), after which some samples were analysed at once whilst the others were incubated in non-radioactive valine in anoxia (chase). Analysis of each sample consisted of complete homogenization in a medium of high pH and ionic strength but which contained no osmoticum; this was followed by differential centrifugation to give sediments at 500 *g* and 64000 *g*, and a final supernatant. The ^{14}C that had been incorporated into protein in each fraction was determined. The sediment at 500 *g* is ascribed to cell wall fragments, that at 63000 *g* to membrane fragments, and the final supernatant to soluble protein from the cytosol and the ruptured organelles. The likelihood of anomalous precipitation of cytoplasmic protein with cell wall fragments was reduced by extracting at pH 7.0 [4] and at high ionic strength [5].

During the pulse there was extensive incorporation of ^{14}C into material that was insoluble in trichloroacetic acid. Almost all of this incorporation was into the cell-wall and the membrane fractions (Table 2). We investigated whether this incorporation was anomalous [6]. The precipitates obtained by adding trichloroacetic acid to the cell-wall and membrane fractions were subjected to both partial and complete hydrolysis. Chromatography of the hydrolysates showed that of the ^{14}C present in the precipitates, 83% and 85% was recovered as [^{14}C]valine in complete hydrolysates from the cell-wall and membrane fractions, respectively. Chromatograms of the partial hydrolysates showed ninhydrin-positive material as a series of discrete bands spread between the origin and the solvent front. These almost certainly represented mixtures of peptides produced by the partial hydrolyses. The ^{14}C originally present in the precipitates was found to be spread throughout these bands. Thus the label in the precipitates was largely present as [^{14}C]valine distributed throughout polypeptide chains, and is unlikely to have been due to exchange of [^{14}C]valine with terminal residues, or to non-specific absorption onto material insoluble in trichloroacetic acid.

The high proportion of anoxically synthesized protein recovered in the membrane fraction at the end of the pulse could have reflected nascent protein on the endoplasmic reticulum en route to some other part of the cell. The fact that the proportion of anoxically formed protein recovered in the membrane fraction did not alter significantly during a chase as long as 48 hr suggests that this is not so, and that the bulk of the anoxic proteins in *Typha* shoots are either attached to membranes or to the cell wall. The slight labelling of the soluble fraction (Table 2) is striking. When *Typha* shoots were incubated aerobically in [^{14}C]valine for 6 hr the percentages of the total ^{14}C incorporated into protein, that were recovered in the cell-wall, membrane and soluble fractions, were 47, 34 and 19, respectively. Further, it has been shown that 60–70% of the protein synthesized by aerobic disks of pumpkin mesocarp [7] was recovered in the supernatant obtained at 144000 *g*. The data in Table 2 add to the evidence that shoots of *Typha angustifolia* are capable of appreciable protein synthesis in anoxia but suggest that this is not a general synthesis of all the proteins needed for normal growth.

The young shoots of *Typha angustifolia* differ from other plant tissues that have been examined in comparable detail, in that in anoxia and hypoxia they are capable of appreciable conversion of sucrose to the polymers re-

Table 1. Effects of oxygen concentration on metabolism of [U-^{14}C]sucrose by excised shoots of *Typha angustifolia*

	Oxygen concentration (%, v/v)								
	21.0	14.3	11.3	8.0	6.1	3.9	2.0	1.0	0
Fresh wt of shoot (mg)	2226	1184	1834	1608	1622	1814	1853	1048	2005
^{14}C metabolized (dpm × 10^{-3})	203.4	172.5	154.9	138.9	134.1	190.7	151.9	156.7	180.1
Percentage of metabolized ^{14}C in:									
CO_2	15.3	12.7	7.5	12.7	2.3	2.2	17.6	11.1	0.1
Water-soluble substances									
Acidic components	44.3	54.4	58.4	60.0	66.4	56.7	54.1	66.4	57.3
Basic components	10.8	9.6	9.1	10.5	9.2	14.9	9.2	9.6	28.9
Water-insoluble ethanol-soluble substances	6.9	6.2	4.8	5.2	7.5	7.2	5.0	3.7	7.0
Water- and ethanol-insoluble substances	22.8	17.1	20.2	10.2	14.6	19.0	14.2	9.2	7.0
Protein (hydrolysis of Pronase)	5.6	6.9	7.5	3.8	5.2	4.5	6.8	2.8	0.9
(hydrolysis by HCl)	5.0	4.5	6.0	4.6	4.4	3.0	5.8	2.1	0.6
Starch	2.6	3.3	2.7	2.7	4.4	5.1	2.5	1.6	2.1
Acidic material solubilized by amyloglucosidase + α-amylase	2.0	1.6	1.6	1.5	2.0	3.1	1.9	1.4	1.5
Pronase and amyloglucosidase-resistant material	4.7	2.1	3.6	2.9	4.9	4.4	4.3	3.2	1.5

Table 2. Intracellular distribution of protein labelled by incubation of excised shoots of *Typha angustifolia* in [U-^{14}C]valine in anoxia

Experiment	Treatment	Total ^{14}C incorporated into protein (dpm × 10^{-3} per sample)	Percentage of total ^{14}C incorporated into protein recovered in:		
			500 *g* sediment	64 000 *g* sediment	64 000 *g* supernatant
1	6 hr pulse	12.0	36	59	5
2	8 hr pulse	22.8	36	63	1
	8 hr pulse: 24 hr chase	55.4	46	52	2
3	8 hr pulse	7.7	19	72	9
	8 hr pulse: 48 hr chase	30.4	35	60	5
4	8 hr pulse	7.8	42	56	2
	8 hr pulse: 48 hr chase	36.5	30	66	4

Shoots, as nearly duplicate as possible for pulse and chase, were incubated in nitrogen for 45 min, then [^{14}C]valine was added for the pulse, at the end of which it was replaced by valine for the chase, samples were homogenized, and centrifuged at 500 *g* (10 min) and at 63 000 *g* (30 min).

quired for growth. This feature of the *Typha angustifolia* shoots may be characteristic of parts of wetland plants that do not owe their ability to tolerate flooding to aerenchyma. Our observation that this capacity for biosynthesis in anoxia and hypoxia may be limited, compared to that in air, raises the possibility that it is restricted to that required to support sufficient cell expansion to allow the shoot to reach a less hypoxic environment. The biochemical basis for hypoxic and anoxic biosynthesis in *Typha angustifolia* shoots is not known, but it is not the use of malate as a major product of fermentation. Analysis of the acidic components of the water-soluble substances of shoots fed [U-^{14}C]sucrose in air, and in nitrogen (Table 1), showed that only 8 and 5% respectively, of the ^{14}C in these fractions was in malate.

EXPERIMENTAL

Materials. Isotopes were from the Radiochemical Centre, Amersham, U.K. and the gas mixtures from P.K. Morgan Ltd., Rainham, Kent, U.K. Enzymes and substrates were from Boehringer. Shoots, plus a length (up to 10 cm) of the rhizomes to which they were attached were taken from plants of *Typha angustifolia* L. growing naturally (The Brick Pits, Wicken Fen, Cambs.). They were taken to the laboratory in pond water in an air-tight box where the shoots were excised and used within 2 hr of being taken from their natural environment.

Metabolism of [^{14}C]*sucrose.* The expts described in Table 1 were carried out essentially as described for roots of *Glyceria maxima* in ref. [1]. Each sample was a single shoot suspended in 5 ml 0.02 M KH_2PO_4, pH 5.2, in a 250-ml Erlenmeyer flask fitted with a side-arm, closed with a self-sealing bung, and inlet and outlet tubes for gassing. Incubation was at 25°. For the first 45 min each sample was gassed with air, nitrogen or mixtures of the two as specified in Table 1. Then 1.0 ml 0.48 mM [U-^{14}C]sucrose (6.25 Ci/mol) was injected into each sample via the self-sealing bung. Gassing was continued throughout the incubation and $^{14}CO_2$ was trapped by passing the outgoing gas through 10% (v/v) KOH. The incubation was stopped, 5 hr after adding the [^{14}C]sucrose, by injecting $HClO_4$ to give a final concn of 1.94 M. Gassing and collection of $^{14}CO_2$ was continued for 45 min: then the sample and its suspending medium were added to liquid N_2, crushed and extracted at 4° with 1.41 M $HClO_4$ (4 × 15 ml). The extracts were combined and freed of perchlorate to give the water-soluble substances that were analysed by ion-exchange and paper chromatography [1]. The water-insoluble material was next homogenized in H_2O, and then extracted, successively, with boiling 80%, 40%, 20% EtOH and H_2O: these extracts were combined to give the water-insoluble ethanol-soluble substances. The residue is the water- and ethanol-insoluble material. Portions of the latter were treated with amyloglucosidase, and α-amylase as in ref. [8] except that incubation was for 15 hr, 156 units of amyloglucosidase were used, and after incubation the reaction mixture was centrifuged (2400 *g*, 10 min) and the supernatant fractionated by ion-exchange chromatography: ^{14}C recovered in the neutral fraction is attributed to starch. The material that resisted solubilization by amyloglucosidase and α-amylase was next suspended in 1.0 ml 50 mM Tris–HCl that contained 7480 units of Pronase (Boehringer) and incubated at 37° for 18 hr. The Pronase treatment was repeated until no further ^{14}C was solubilized. The label that was solubilized by Pronase, plus that recovered in the basic components of the hydrolysate produced by amyloglucosidase and amylase, were summed and attributed to protein. The validity of this procedure is demonstrated by the fact (Table 1) that the estimate of ^{14}C in protein obtained was similar to that found when the protein was hydrolysed by HCl and the labelling of the resulting amino acids determined as in ref. [1].

For experiments with shoots attached to their parent rhizomes, we put a shoot attached to a 1-cm length of rhizome in a 500-ml flask filled with culture soln (double strength that in ref. [9]) that was gassed for 7 or 3 days with 95% N_2–5% CO_2. The shoot projected into a small tube that was within the 500-ml flask but also connected to the outside via a self-sealing bung. After the 7 or 3 day pretreatment, the culture soln was removed and 22 ml 0.6 M [U-^{14}C]sucrose (0.05 Ci/mol) was injected into the tube that contained the shoot so that, although the shoot was still attached to the rhizome, only the shoot was immersed in the [^{14}C]sucrose. After 6 hr in the labelled sucrose the expt was stopped by the addition of $HClO_4$ to 1.76 M and the samples analysed as described above. Gassing was continued throughout the expt, which was done at 25°.

Metabolism of [^{14}C]*valine.* Shoots were prepared and incubated as for the feeding of [^{14}C]sucrose to excised shoots (Table 1) except that the initial suspending medium was 8 ml 0.02 M KH_2PO_4, pH 5.2. Samples were gassed with oxygen-free

nitrogen for 45 min and then 0.5 ml 0.2 mM [U-^{14}C]valine (75 Ci/mol) was injected into each flask. Gassing was continued throughout the pulse and chase. Six or eight hr after the addition of [^{14}C]valine the pulse samples were fractionated at once but the chase samples were rapidly rinsed in 4.2 mM valine in 0.02 M KH_2PO_4, pH 5.2, and then resuspended in 8.0 ml of this soln for the chase. Samples taken for analysis at the end of the pulse and the chase were rinsed with 4.2 mM valine in 0.02 M KH_2PO_4, pH 5.2, and then chopped with razor blades and homogenized with a pestle and mortar and an all-glass homogenizer in 10 vol 4.2 mM valine in 1 M KH_2PO_4, pH 7.0 (extraction medium). The homogenate was centrifuged at 500 *g* for 10 min and the resulting pellet was resuspended in extraction medium and centrifuged at 3500 *g* for 10 min. The two supernatants were combined and centrifuged at 63 000 *g* for 30 min to give what we call sediment at 63 000 *g* and supernatant at 63 000 *g*. The material that sedimented at 500 *g* and again at 3500 *g* was washed (7 × 30 ml extraction medium) by resuspension and centrifugation at 3500 *g* to give what we call the 500 *g* sediment. The above fractionation was done quickly at 2°. As soon as the final fractions were obtained each was made 5% (w/v) with trichloroacetic acid. The resulting ppts were washed according to ref. [7] and counted. Complete and partial hydrolysis of protein was as in ref. [7].

General. ^{14}C in aq. samples was measured as in ref. [1] or after adding Beckman 'Ready Solv' EP scintillation fluid. ^{14}C in insoluble material was determined as in ref. [1] after treatment with NCS Tissue solubilizer (BDH Poole, Dorset, U.K.). Redox potentials were measured as in ref. [2], and oxygen in pond water with a portable oxygen meter (E.I.L. Analytical Instruments, Richmond, Surrey, U.K.).

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