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GLUCONEOGENESIS DURING THE GERMINATION OF *CUCURBITA PEPO*

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Key Word Index—*Curcurbita pepo*; Cucurbitaceae; marrow; gluconeogenesis; germination; sucrose; stachyose; fructose-1,6-diphosphatase; glyceraldehyde-3-phosphate dehydrogenases.

Abstract—The contents of lipid and sugar, the distribution of ^{14}C from [2-^{14}C]acetate, and the activities of fructose-1,6-diphosphatase (E.C. 3.1.3.11), NAD- (E.C. 1.2.1.12) and NADP- (E.C. 1.2.1.13) linked glyceraldehyde-3-phosphate dehydrogenases, were determined in seeds and in cotyledons of marrow (*Cucurbita pepo* L. var. *medullosa* Alef.) germinated in the dark for 2, 5, and 8 days. Gluconeogenesis was shown to be slight for the first 2 days of germination and to be appreciable for the next 6 days. Sucrose and stachyose were the major products of a gluconeogenesis that was accompanied by substantial labelling, by [2-^{14}C]acetate, of the fractions that contained lipids, amino acids, nucleotides, and protein. Fructose-1,6-diphosphatase activity increased in parallel with the labelling of sugars by [2-^{14}C]acetate. It is suggested that variation in this enzyme contributes to the control of gluconeogenesis. The activity of NAD-linked glyceraldehyde-3-phosphate dehydrogenase rose during germination whilst that of the NADP-linked enzyme was, by comparison, negligible at all stages. It is suggested that the NAD-linked enzyme is responsible for glyceraldehyde-3-phosphate formation in gluconeogenesis.

INTRODUCTION

THE GERMINATION of castor bean is accompanied by massive gluconeogenesis from lipid.[1] Studies with other plants,[2–5] and comparative biochemistry, strongly suggest that the pathways demonstrated in castor bean represent the mechanism of lipid mobilization during germination in general. Part of the pathway in this gluconeogenesis is the reverse of the glycolytic sequence from hexose phosphate to phosphoenolpyruvate.[6] Glycolysis appears to be a universal feature of tissues of higher plants.[7] This poses the question of the relationship between gluconeogenesis and glycolysis during the germination of fatty seeds. We think this is important: (1) The gluconeogenic and glycolytic reactions between phosphoenolpyruvate and hexose phosphate appear as an exception to the general observation that anabolic and catabolic pathways are separate. (2) Control of the two pathways is important in energy conservation as their co-existence could lead to 'futile cycles' that act as net ATPases at a critical point in the development of the plant.[8] (3) The relative activities of the two pathways could profoundly affect the utilization of storage fat.

We have investigated the above problem in the cotyledons of marrow (*Cucurbita pepo* L. var. *medullosa* Alef.) We chose these cotyledons because they persist as photosynthetic tissues in the seedling. Thus, in contrast to castor bean, the period of gluconeogenesis is

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followed by differentiation of gluconeogenic tissue into mature respiring tissue. Our approach was to assess the relative activities of gluconeogenesis and glycolysis at different stages of germination in order to see whether developmental changes would reveal anything of the relationship between the two pathways. We made these assessments by measuring net changes in the end products of the pathways, by determining the distribution of label from key intermediates, and by measuring the activities of selected enzymes. In this paper we report on the development of gluconeogenesis in cotyledons of seedlings germinated in the dark. [2-^{14}C]Acetate was chosen as the labelled intermediate because movement of label from this compound into sugars is a good indication of gluconeogenesis. Fructose-1,6-diphosphatase (E.C. 3.1.3.11) was chosen because it is characteristic of gluconeogenesis but not glycolysis. Glyceraldehyde-3-phosphate dehydrogenase was selected because it catalyses an energetically important step and because a comparison of activity with NAD (E.C. 1.2.1.12) and NADP (E.C. 1.2.1.13) might indicate the source of the reducing power used in gluconeogenesis.

RESULTS

Changes in Lipid and Sugar

Table 1 shows the general features of the different stages of germination that we examined. These data allow the results in subsequent tables to be considered in relation to each of these features throughout germination. Changes in lipid and sugar contents are in Table 2. The values for sugar represent the reducing sugar in the water-soluble fraction of

TABLE 1. GENERAL FEATURES OF GERMINATING MARROW SEEDS

Days germinated	Stage of development	Fr. wt (mg/cotyledon)	Fr. wt (mg/seedling)	Extracted protein* (mg/cotyledon)	Cells per* cotyledon $\times 10^{-6}$
0	Dry seed	56	103	0·84 ± 0·02 (6)	5·91 ± 0·75 (4)
2	Radicle 1–5 mm long	70	137	1·50 ± 0·08 (6)	3·97 ± 0·68 (5)
4	Cotyledons within testa but above ground	—	254	—	—
5	Cotyledons free of testa	99	—	8·30 ± 0·22 (6)	5·32 ± 0·56 (4)
6	Cotyledons open	—	881	—	—
8	Cotyledons expanded, hypocotyl elongated, plant etiolated	121	1054	6·98 ± 0·10 (6)	6·59 ± 1·1 (4)

* Values are means ± S.E. The number of batches of seedlings assayed is given in parentheses.

the tissues after these fractions had been treated with invertase. The most important aspect of these results is the coincidence between the fall in lipid and the rise in sugar that occurred between 2 and 6 days from the start of germination. It should be noted that there is no evidence of gluconeogenesis in the first 2 days of germination.

Distribution of Label from [2-^{14}C]*acetate*

For each stage of germination, duplicate samples of cotyledons were incubated in [2-^{14}C]acetate. One member of each pair was used to determine incorporation of ^{14}C into

TABLE 2. LIPID AND SUGAR CONTENTS OF GERMINATING MARROW SEEDS

Days germinated	Lipid* (g/100 seedlings)	Sugar* (mg/100 seedlings)
0	4·82	81
2	4·66	45
4	4·34	227
6	2·02	895
8	2·20	894

* Data refer to seeds or seedlings without testae.

lipids and the other was used to determine the labelling of the other fractions shown in Table 3. At the end of the incubation the medium was analysed in order to see whether labelled metabolites had leaked from the tissue. Unmetabolized [2-^{14}C]acetate was removed from the water-soluble components of the tissue and from the medium before these solutions were analysed. The amount of [2-^{14}C]acetate metabolized is the sum of the ^{14}C recovered in the water-soluble components of the tissue and the medium, after removal of unmetabolized [2-^{14}C]acetate, plus the ^{14}C recovered in the ethanol-insoluble fraction, CO_2, and lipids. Losses of ^{14}C during the ion-exchange chromatography were always less than 5% of the ^{14}C present in the unfractionated water-soluble components of the tissue. The amounts of metabolized [2-^{14}C]acetate recovered in the media at the end of the incubations were 12, 6, 4, and 2% for seeds and 2-, 5-, and 8-day-old cotyledons, respectively. The labelling of the neutral, basic, organic acid, and nucleotide fractions of the media reflected that of the water-soluble components of the tissue.

TABLE 3. DISTRIBUTION OF ^{14}C RECOVERED FROM MARROW COTYLEDONS SUPPLIED WITH [2-^{14}C]ACETATE

Cell fraction	% metabolized ^{14}C recovered per fraction during germination (days)			
	0	2	5	8
CO_2	1·1	3·4	6·2	7·0
Lipid	7·0	10·8	16·2	10·6
Water soluble components*				
Neutral fraction	1·7	1·1	14·3	11·0
Basic fraction	45·5	37·4	15·3	22·0
Organic acids	27·9	20·0	14·0	24·0
Nucleotides	5·8	4·0	3·1	4·3
Material insoluble in 80% ethanol	18·0	24·5	28·4	19·1

* Figures for these fractions represent ^{14}C recovered in tissue plus that recovered in the medium at the end of the incubation.

We emphasize the following aspects of the metabolism of [2-^{14}C]acetate (Table 3). Firstly, there was considerable conversion of acetate to lipid that reached a peak in 5-day-old cotyledons. Secondly, the heaviest labelled fractions were those that contained the organic acids, the amino acids, and the proteins. Labelling of the fractions that contained the amino acids was appreciably higher during the first 2 days of germination than subsequently.

Finally, only a very small proportion of the metabolized [2-^{14}C]acetate was found in the neutral fractions from seeds and 2-day-old cotyledons. This contrasts sharply with the substantial labelling of the neutral fraction from 5- and 8-day-old cotyledons. Evidence that the ^{14}C in these neutral fractions was mainly in sugars is provided in Table 4. Sugars, isolated from the neutral fractions by paper chromatography, contained at least 80% of the label present in these fractions. Although sucrose was the most heavily labelled sugar, there were considerable amounts of label in stachyose.

TABLE 4. LABELLING OF SUGARS OF MARROW COTYLEDONS BY [2-^{14}C]ACETATE

	% ^{14}C in neutral fraction* of aqueous extract recovered per sugar during germination (days)		
Sugar	0	5	8
Stachyose	9	20	30
Raffinose	1	4	5
Sucrose	57	48	35
Glucose	7	10	7
Fructose	4	9	5
Galactose	1	1	2

* This fraction is the neutral fraction of the water soluble components described in Table 3.

Enzyme Studies

We found that the pH optima for fructose-1,6-diphosphatase activity in extracts of seeds, and of 2-, 5-, and 8-day-old cotyledons were within the values pH 7·7–7·9, 7·2–7·5, 7·0–7·2, and 6·8–7·0, respectively. Thus we measured fructose-1,6-diphosphatase activity at pH 7·8, 7·4, 7·0, and 7·0 in extracts of seeds and of 2-, 5-, and 8-day-old cotyledons, respectively (Table 5). A marked rise in activity occurred during germination, with the greatest increase occurring between days 2 and 5. To check that the differences in activity in Table 5 were not due to failure to assay the enzyme under optimum conditions, we studied the effects of varying, independently, the concentrations of each component of the assay

TABLE 5. ACTIVITY OF FRUCTOSE-1,6-DIPHOSPHATASE IN EXTRACTS OF MARROW COTYLEDONS DURING GERMINATION

Days germinated	Activity* (nmol substrate consumed/ cotyledon/min)	Fisher's $\bar{P}$ values†	
0	14·7 ± 1·4	0 vs. 2	<0·002
2	40·5 ± 6·1	2 vs. 5	<0·001
5	321 ± 24	5 vs. 8	N.S.
8	332 ± 19	2 vs. 8	<0·001

* Values are means ±S.E. of activities of 6 different extracts.

† Fisher's $\bar{P}$ values are given for comparison of activities. Values of 0·05 or less are considered significant. Values greater than 0·05 are given as N.S. (not significant).

mixtures on the activity in extracts of seeds and of 5-day-old cotyledons. The results showed that the assay mixtures used to obtain the data for these extracts in Table 5 gave the highest activities. The differences in the activities of extracts of cotyledons of different ages could have been due to inhibition or activation of the enzyme during the preparation of the extracts. We tested this possibility by comparing activities in extracts of seeds, and in extracts of 8-day-old cotyledons, with the activity in an extract of a sample composed of a mixture of equal weights of cotyledons from seeds and from 8-day-old plants. We carried out a similar experiment with 2- and 5-day-old cotyledons. In both types of experiment we found that the activities of the mixed samples were 97–100% of the activities predicted from the measurements made on the separate samples.

We assayed glyceraldehyde-3-phosphate dehydrogenase in the forward (glyceraldehyde-3-phosphate → 1,3-diphosphoglyceric acid) and reverse directions with NAD and with NADP (Table 6). Activity with NADP was very low, both in absolute terms and in relation to the activity with NAD. Activity with NAD rose markedly during the first 2 days and then declined after 5 days. In contrast to the activities with NADP, those with NAD were higher in the reverse than in the forward direction. We studied the effects of varying, independently, the concentrations of the components of the assay mixtures on the activities in extracts of seeds and of 5-day-old cotyledons. This was done for assays in both directions and with both NAD and NADP. Optimum activities were found under the conditions used to obtain the data in Table 6. The possibility that inhibition or activation during the preparation of the extracts affected our measurements of the NAD-linked activity was considered. We compared the activities of a commercially purified enzyme, of untreated extracts, and of extracts to which measured amounts of the purified enzyme had been added. We did this with extracts of cotyledons of all the ages studied. No extract had any significant effect on the activity of the purified enzyme.

TABLE 6. ACTIVITY OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE IN EXTRACTS OF MARROW COTYLEDONS DURING GERMINATION

Days germinated	Activity* (nmol substrate consumed/cotyledon/min) with the following substrates: NAD+	NADH	NADP+	NADPH
0	260 ± 13	447 ± 56	2·5 ± 0·1	0
2	604 ± 21	1045 ± 132	3·3 ± 0·9	3·3 ± 0·7
5	940 ± 145	1475 ± 297	9·2 ± 2·0	11·3 ± 3·0
8	470 ± 89	546 ± 177	14·3 ± 4·0	12·7 ± 4·0
Comparison	Fisher's $\tilde{P}$ values†			
0 vs. 2	<0·001	<0·002	N.S.	—
2 vs. 5	N.S.	N.S.	N.S.	<0·05
5 vs. 8	<0·05	<0·05	N.S.	N.S.
0 vs. 5	<0·002	<0·01	<0·05	—
0 vs. 8	<0·05	N.S.	<0·05	—

* Values are means ±S.E. of activities of 6 different extracts for assays in the forward direction and of 5 different extracts for assays in the reverse direction.

† Fisher's $\tilde{P}$ values are given as in Table 5.

DISCUSSION

The cell counts show that little cell division occurred in the cotyledons during germination. Thus the changes in metabolism that we found represent differentiation of the cells that were present in the seeds. Our estimates of lipid and sugar indicate that there was little gluconeogenesis from fat in the first 2 days of germination and that such activity developed thereafter to become most extensive after 4–6 days germination. Storage fat may have declined more rapidly and completely than is indicated in Table 2 as differentiation of the cotyledons during germination almost certainly involved the synthesis of lipids. This view is supported by the extensive movement of label from [2-^{14}C]acetate into the lipid fraction (Table 3) and also by the evidence that there is a net synthesis of phosphatide in the cotyledons of a related plant, watermelon, during germination.[9] From Table 2 it can be seen that the accumulation of sugar does not account for all the lipid broken down. Three features may contribute to this discrepancy. Some sugar must have been used to support the growth of the seedling. Not all of the acetyl-CoA produced from lipids may have been converted to sugars. Finally we almost certainly underestimated the sugar content of the seedlings, since treatment of stachyose with invertase would not have converted it to its monomers but would have yielded fructose and the trisaccharide manninotriose.[10]

We have used our measurements of lipid and sugar to estimate the maximum and minimum rates of gluconeogenesis. Using the fatty acid composition of the storage fat of another variety of *Cucurbita pepo*,[11] we calculate that about 9 mol of hexose would be formed from 4 mol of fatty acid via the scheme described by Beevers.[1] An indication of the maximum rate of gluconeogenesis can be obtained by assuming that all the fat in a 4-day-old seedling was converted to sugar by day 6. This would give a rate of 60 nmol hexose formed per min per cotyledon. A minimum rate of gluconeogenesis of 6·4 nmol hexose formed per min per cotyledon is obtained by assuming that the net increase in sugar between days 4 and 6 is due to gluconeogenesis.

The labelling of sugars by [2-^{14}C]acetate indicates that gluconeogenesis was slight for the first 2 days of germination but increased rapidly between days 2 and 5 to become a significant activity of the cotyledons. The considerable labelling of stachyose, as well as that of sucrose, and the slight labelling of raffinose, may be correlated with the roles that these compounds play in translocation in the Cucurbitaceae.[12,13] This labelling pattern, and the predominant labelling of sucrose as opposed to hexose during gluconeogenesis in castor bean,[14] peanut, and sunflower,[2] indicate that the prime significance of gluconeogenesis during the germination of fatty acids is the conversion of the storage material into the relatively restricted group of non-reducing compounds used by plants to translocate carbon.

The distribution of ^{14}C from [2-^{14}C]acetate during gluconeogenesis by marrow cotyledons differs from that found in castor bean endosperm,[14] the only other tissue in which such a detailed analysis has been made. Firstly, much less ^{14}C accumulated in sugars in marrow. Secondly, the labelling of the lipid and ethanol-insoluble fractions was much higher in marrow than in castor bean. These differences can be explained by the fact that castor bean endosperm is senescent after gluconeogenesis whilst marrow cotyledons develop into photosynthetic tissues. In the endosperm of 5-day-old castor bean seedlings

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gluconeogenesis is probably by far the dominant process using acetyl-CoA. In marrow cotyledons gluconeogenesis must be accompanied by extensive use of acetyl-CoA in the synthesis of amino acids, nucleotides, proteins, nucleic acids and lipids. Although the analyses are less complete than those mentioned above, the distribution of label from [^{14}C]acetate supplied to other gluconeogenic tissues that persist after the fat has been used show similarities to the pattern obtained with marrow cotyledons.[2,4,5] The marked movement of ^{14}C from [2-^{14}C]acetate into the fractions that contained the amino acids, proteins, nucleotides, and nucleic acids, that occurred during the first 2 days of germination in marrow, probably represents the synthesis of protein and nucleic acid responsible for subsequent differentiation.

We think that our estimates of enzyme activities reflect the catalytic abilities of the cotyledons. The failure of the extracts to affect the activity of purified glyceraldehyde-3-phosphate dehydrogenase, and the close agreement between the actual and predicted values for fructose-1,6-diphosphatase in extracts of mixtures of cotyledons of different ages, strongly indicate that appreciable inhibition or activation of the enzymes did not occur during the preparation of the extracts. Our examination of the conditions of assay indicate that the major changes in activity were not due to any failure to assay the enzymes under optimum conditions.

As fructose-1,6-diphosphatase is characteristic of gluconeogenesis, the data in Table 5 indicate the extent of gluconeogenesis at different stages of germination. The activity of fructose-1,6-diphosphatase correlates closely with the labelling of sugars by [2-^{14}C]acetate, and with the changes in lipid and sugar. The very good agreement between these three different types of experiment leads us to conclude that there was little or no gluconeogenesis from fat during the first 2 days of germination and that such gluconeogenesis became extensive between days 2 and 5 and continued at least until day 8. Measurements, at different stages of germination, of enzymes involved in the conversion of fat to sugar have been reported for a number of fatty seeds (see Ref. 15). Although these measurements have not been supported by feeding experiments at each developmental stage, the results strongly indicate that the development and decline of gluconeogenesis is a general feature of the germination of fatty seeds. The studies reviewed in the above reference also indicate that the changes in gluconeogenesis are due to the formation and loss of at least some of the enzymes involved. The extent to which fructose-1,6-diphosphatase and glyceraldehyde-3-phosphate dehydrogenase contribute to such coarse control of gluconeogenesis in marrow cotyledons is considered below.

Fructose-1,6-diphosphatase activity has been shown to be associated with gluconeogenesis in non-photosynthetic tissues of higher plants.[16–18] Castor bean endosperm has an AMP-sensitive enzyme (optimum pH 7·5) and an AMP-insensitive enzyme (optimum pH 6·7) and it is the latter that increases during germination. The changes in pH optima that we found could reflect a similar situation in marrow cotyledons. We did not fractionate fructose-1,6-diphosphatase activity in extracts of marrow because we wished to avoid any loss of enzyme in our comparison of total activity with our estimates of the rate of gluconeogenesis. When this is done it can be seen that the enzyme activity up to 2 days of germination is barely adequate to support even our minimum estimate of the rate of gluconeogenesis.

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This, and the close parallel between the enzyme activity and the labelling of sugars by [2-^{14}C]acetate, indicate that changes in the maximum catalytic activity of fructose-1,6-diphosphatase contribute to the regulation of gluconeogenesis in marrow cotyledons.

Wu and Racker,[19] working with extracts of Ascites tumour cells, also found higher activity of glyceraldehyde-3-phosphate dehydrogenase in the reverse than in the forward direction. Whichever direction of assay of the NAD-linked activity is considered, two points are clear. First, cotyledons at all stages contained enough activity to support the maximum estimate of gluconeogenesis. There is thus no evidence that this enzyme exerts coarse control over gluconeogenesis. Second, the activity rose significantly during the first 2 days germination but this rise was not closely correlated with gluconeogenesis. This may reflect the presence of two enzymes, one for glycolysis, and one, with an equilibrium favouring 1,3-diphosphoglycerate reduction, for gluconeogenesis. Support for the argument that an NAD-, rather than an NADP-, linked enzyme catalyses the reductive step in gluconeogenesis is provided by the contrast between the two activities (Table 6). The activities with NADP were insufficient to support even our minimum estimate of gluconeogenesis. Finally we stress that ample $NADH_2$ for gluconeogenesis could be provided by the reactions of the glyoxysome.[20]

EXPERIMENTAL

Material. Seeds of vegetable marrow (*Cucurbita pepo* L. var. *medullosa* Alef.) of the variety Long Green Bush were surface sterilized in 7% (v/v) sodium hypochlorite for 15 min, washed in running tap water for 3 hr, soaked in distilled water at 25° for 4 hr, planted at a depth of 2·5 cm in autoclaved vermiculite, and germinated in the dark at 25°. The vermiculite was watered at the time of planting and at 2-day intervals thereafter. Time of germination is measured from the end of the surface sterilization. Samples of ungerminated seeds, and of 2-day-old cotyledons consisted of seeds minus testae. Samples of ungerminated seed were prepared immediately after the dry seeds had been soaked in water at 2° for 10 min. Cotyledons were severed at their base from seedlings. The time between the removal of the seedlings from the darkened growth chamber to the beginning of the experimental treatments was 5 min for preparation of extracts for enzyme assays and 30 min for the feeding experiments.

Measurement of lipid and sugar. Duplicate samples (fr. wt 5–12 g) of seeds, and of seedlings at each stage of germination, were prepared. One member of each pair of samples was used for measurement of lipid and the other for measurement of sugar. Lipid was extracted and assayed as described by Willemot and Stumpf.[21] For estimation of sugar, the samples were killed in boiling 80% (v/v) aq. ethanol and extracted with the same solution in a Soxhlet apparatus for 12 hr. The ethanol-soluble fraction was reduced to dryness and then extracted successively with chloroform and water. The water-soluble components were treated with invertase and assayed for reducing sugars as described previously.[22]

Metabolism of [2-^{14}C]*acetate.* Unnecessary slicing and the consequent risk of induced respiration[23] were avoided. The following procedure involved the minimum amount of slicing that was consistent with significant uptake of isotope and an equal distribution of label throughout the pieces of tissue. Strips 1–2 mm wide were cut off the tips and sides of the cotyledons and discarded. The remainder of each cotyledon was quartered and replicate samples, each of 80 quarter-cotyledons, were prepared. Each sample was incubated for 4 hr in the dark at 25° in a 100 ml Erlenmeyer flask and was suspended in 5·0 ml 0·02 M KH_2PO_4 (pH 5·2) that contained 8 μCi [2-^{14}C]acetate at 0·25 mM. $^{14}CO_2$ was collected in KOH in the centre well. At the end of the incubation the medium was removed, the tissue was washed twice with 2·5 ml portions of 0·02 M KH_2PO_4 (pH 5·2) and the washings were added to the medium. For measuring the labelling of lipid, the samples were killed and extracted as described for lipid analysis above. For determining the labelling of all other fractions, the samples were killed and extracted as described above for the sugar assays. The water-soluble components of the ethanol extracts were also prepared as described above. In order to remove unmetabolized [2-^{14}C]acetate from the extract excess (50 ml) glacial acetic acid was added to each water-soluble extract. The extract was then evaporated to dryness *in vacuo* at 30° and the residue was redissolved in water. The cycle of evaporation and solution was repeated four times in order to remove all the acetate

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before the extract was fractionated by ion-exchange chromatography as described by Fowler and ap Rees.[24] The medium was reduced to dryness, treated with acetic acid and fractionated as described for the water-soluble components above. Portions of the neutral fractions of the water-soluble components were analysed by PC in ethyl acetate–pyridine–H_2O (8:2:1) for 26 hr. This isolated mono- and di-saccharides but left raffinose and stachyose at the origin. After this first chromatography, the material at the origin was eluted and analysed by paper chromatography in butanol–pyridine–H_2O (6:4:3) for 60 hr. This isolated both stachyose and raffinose. The radioactivity of the isolated sugars was determined, after they had been eluted from the chromatograms, by assay in a Tracerlab Coru/Matic II liquid scintillation spectrometer. The scintillation fluid was a mixture of toluene and Triton X-100 (2:1). The toluene contained 2,5-diphenyl-oxazole (0·4%, w/v) and 1,4-bis-(5-phenyl-oxazol-2-yl) benzene (0·01%, w/v). Counting efficiency was 78%. All other measurements of ^{14}C were after conversion to $Ba^{14}CO_3$ as described previously.[25]

Assay of enzymes. Samples of cotyledons (fr. wt 5 g) were homogenized first with a pestle and mortar and then with a glass homogenizer in 20 ml 40 mM glycylglycine buffer (pH 7·4). Microscopic examination of the extracts showed that this procedure left very few unbroken cells. The homogenate was centrifuged for 30 min (70 000 *g*) and the supernatant was used at once for the enzyme assays. The extracts were prepared at 4° and assayed at 25°. Fructose-1,6-diphosphatase was assayed according to Racker[26] except that the buffer was 0·1 M glycylglycine. Glyceraldehyde-3-phosphate dehydrogenase was assayed according to Wu and Racker[19] with the following alterations. The buffer was 0·1 M glycylglycine (pH 7·4); in the oxidative direction the final concentrations of NAD(P) and sodium arsenate were 3 and 30 mM, respectively, and in the reductive direction 3·6 units of phosphoglycerate kinase were used in the final volume of 3·0 ml. Protein was precipitated with 5% (w/v) trichloroacetic acid and assayed as described previously.[24] Cell counts were made by the method of Humphries and Wheeler.[27]

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