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SUCROSE PARTITIONING IN DEVELOPING EMBRYOS OF ROUND AND WRINKLED VARIETIES OF *PISUM SATIVUM*

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Key Word Index—*Pisum sativum*; Leguminosae; pea; embryos; [^{14}C] sucrose metabolism; sucrose synthase; invertase.

Abstract—This work is an investigation of the roles of sucrose synthase, acid invertase and alkaline invertase in sucrose metabolism in developing embryos of cv. Birte and cv. Greenshaft of *Pisum sativum*. The detailed distribution of label from [U-^{14}C]sucrose showed that Birte embryos converted more sucrose to starch and protein than did Greenshaft embryos. From 0.06 to 0.53 M [^{14}C] sucrose, uptake by embryos of both varieties exceeded its metabolism. No acid invertase was found in embryos of either variety. The maximum catalytic activity of sucrose synthase was roughly ten times that of alkaline invertase: both activities rose steadily during development. The rates of uptake and metabolism of [^{14}C]sucrose were used to estimate the rates of sucrose breakdown *in vivo*. The results suggest that both sucrose synthase and alkaline invertase contribute to this breakdown, with the former making the greater contribution.

INTRODUCTION

As sucrose is the principal form in which carbon and energy are supplied to most non-photosynthetic cells of higher plants, the manner of its immediate metabolism is of fundamental significance. This is particularly true of developing seeds of economic importance. Such metabolism is likely to be regulated, in the first instance, by the relative activities *in vivo* of the only three enzymes known to be capable of metabolizing sucrose in higher plants, acid and alkaline invertases (EC 3.2.1.26) and sucrose synthase (EC 2.4.1.13). The relative roles of these enzymes are still not understood, particularly in respect of the conversion of sucrose to starch [1].

The aim of the work reported in this paper was to investigate the roles of the above enzymes in sucrose metabolism in embryos of two varieties of peas that differed widely in their content of the final products of sucrose metabolism, starch in particular. The two varieties, Birte and Greenshaft, differ primarily at the *Ra* locus. Seeds of the former contain appreciably more starch [2] and legumin [3] than those of the latter. We refer to Birte and Greenshaft as round and wrinkled peas, respectively. There is adequate evidence that developing seeds of legumes receive the bulk of their carbon as sucrose that is transported as sucrose into the developing embryo from the surrounding apoplast [4]. Our experimental approach was to select a period of rapid embryo growth and starch synthesis, and then to establish how the embryos partitioned sucrose by analysing the distribution of label after supplying [^{14}C]sucrose. We followed this by estimating the rate of sucrose breakdown from measurements of the uptake and metabolism of [^{14}C]sucrose. These estimates were then compared with measurements of the maximum catalytic activities of the enzymes. We emphasize that the work was done with embryos. Developing seeds were removed from the pod and the testa was removed. The whole of the material within the testa, which at the stages of development studied, consisted almost entirely of cotyledons together with a small radicle, was used without further dissection.

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RESULTS

[^{14}C]*Sucrose metabolism*

Measurements of the fresh weight (Fig. 1) and starch content (Fig. 2) of the embryos showed rapid growth and starch accumulation as the embryos developed from 100 to 400 mg fresh weight. We chose this period of development for our study, and designate as arbitrary stages of development fresh weights of 100, 200, 300 and 400 mg. As it was not always practicable to obtain embryos of these weights precisely, we took embryos that were within 40 mg of the weights. Thus, for example, at the 100 mg stage we used embryos between 60 and 140 mg: for each sample within this range enzyme activity is expressed per 100 mg to give activity per embryo at the 100 mg stage.

To determine how the embryos partitioned sucrose we gave them a 6-hr pulse in [U-^{14}C]sucrose. This was provided in a medium similar to that used for the successful culture of pea embryos [5] but with the [^{14}C]sucrose at a low concentration to give a high specific activity. At the end of the pulse the embryos were transferred to unlabelled culture medium with the usual concentration of sucrose (chase medium), incubated for 24 hr and then analysed in detail (Table 1). At the end of the chase, samples of the chase medium were plated on Difco Bacto nutrient agar and incubated at 30° for 48 hr. Data are presented only for experiments that showed no

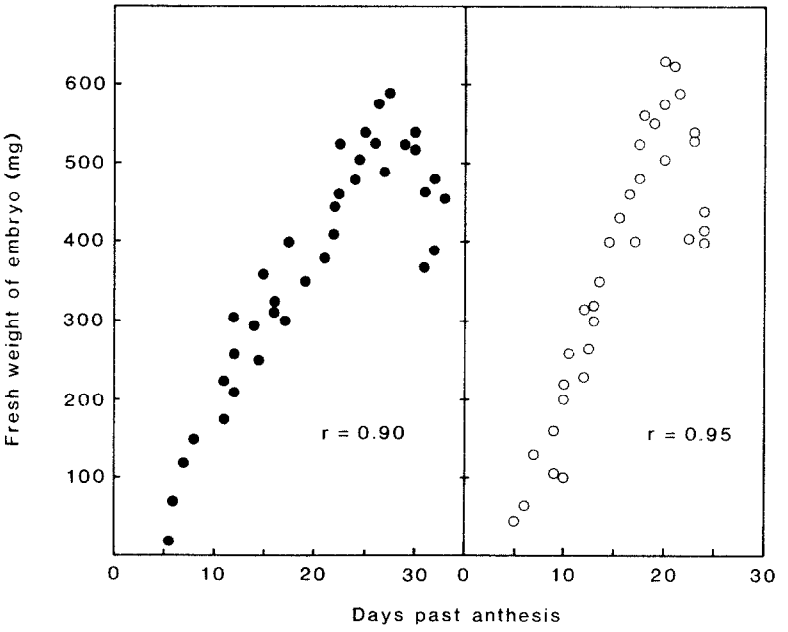

Fig. 1. Changes in fresh weight of embryos of round (●) and wrinkled (○) seeded peas during developmet. Pods were tagged 1 day after anthesis and complete embroys were removed and weighed at intervals thereafter: r is calculated for embryos between 100 and 400 mg fr. wt.

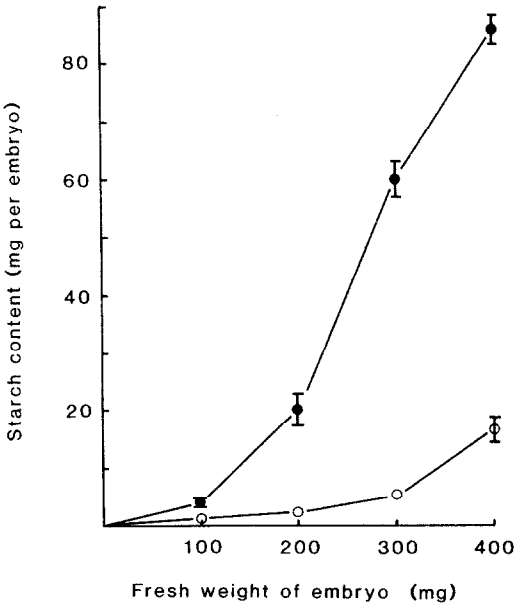

Fig. 2. Starch content of embryos of round (●) and wrinkled (○) seeded peas during development. Each value is the mean of estimates from at lest four different measurements made on four different samples of embryos. The bars show s.e.m. where these are large enough to be shown.

microbial contamination in this test. We report two experiments. In each, a sample of nine embryos was given a pulse and chase, and then divided into three samples, each of three embryos. Each of the latter were analysed separately. Thus our figures for CO_2 production during the incubation (Table 1) are for two samples, each of nine embryos, whereas the figures for all other fractions are means from six samples, each of three embryos. In order to compare different samples, the label in each fraction of a given sample is expressed as a percentage of the total ^{14}C metabolized by that sample. The latter is the sum of the label in $^{14}CO_2$, lipid, water-insoluble material, acidic and basic components of the water-soluble material, glucose, fructose and oligosaccharides other than sucrose (Table 1). For each sample we determined: ^{14}C metabolized, ^{14}C present in the embryos as [^{14}C]sucrose, and ^{14}C remaining in the pulse and chase media. The sum of the latter was at least 89% of the ^{14}C initially supplied in the pulse medium. Thus losses during the extensive analyses were slight.

We stress the following aspects of our analyses (Table 1). First, embryos of round peas metabolized almost twice as much [^{14}C]sucrose as did those of wrinkled peas. Second, in both varieties the basic labelling pattern was that expected of a non-photosynthetic storage tissue, but there were important differences between the varieties. Specifically, embryos of round peas incorporated appreciably more label into insoluble material than did those of wrinkled peas. Most of this was in starch but there was also significantly greater labelling of protein. Conversely, more of the label metabolized by the embryos of wrinkled peas was recovered in CO_2, lipid, and basic and acidic compounds. Collectively, the labelling of the latter fractions represents the proportion of [^{14}C]sucrose that entered the respiratory pathways. This proportion, the sum of the label in the above fractions, amounted to 11.3% more of the metabolized label in wrinkled than in round peas. This difference is close to the difference in the labelling of the insoluble material, 12% of the metabolized label.

To obtain estimates of the rate of sucrose metabolism we incubated embryos in a range of concentrations of

Table 1. Distribution of ^{14}C after supplying [U-^{14}C]sucrose to developing embryos of round and wrinkled peas

Fraction	^{14}C per fraction as % of total ^{14}C metabolized		Fisher's P value for round versus wrinkled
	Round	Wrinkled	
CO_2	6.8	12.5	
Lipid	2.3 ± 0.1	3.7 ± 0.4	< 0.02
Ethanol and water-insoluble material	70.2 ± 0.9	58.0 ± 0.6	< 0.001
Starch	51.9 ± 2.1	39.0 ± 2.1	< 0.01
Protein	14.0 ± 0.7	11.1 ± 0.3	< 0.02
Water-soluble material	20.9 ± 0.7	25.7 ± 1.0	< 0.001
Basic components	5.3 ± 0.2	7.0 ± 0.4	< 0.001
Acidic components	6.2 ± 0.2	8.7 ± 0.5	< 0.001
Glucose and fructose	2.5 ± 0.2	5.1 ± 0.4	< 0.01
Oligosaccharides	6.9 ± 0.5	4.0 ± 0.6	< 0.02
^{14}C metabolized (dpm × $10^6 \cdot g^{-1}$ fr. wt)	3.8 ± 0.1	2.0 ± 0.2	< 0.001

Embryos were incubated in [^{14}C]sucrose for 6 hr and then in sucrose for 24 hr. Values are means ± s.e.m. from six samples of embryos.

[U-^{14}C]sucrose in culture media for 6 hr, and measured uptake, total metabolism and metabolism to insoluble material. We varied the concentration of sucrose to see if the uptake and the extent of its metabolism varied with its concentration and with the type of embryo. Uptake of [^{14}C]sucrose was linear with respect to time for longer than 6 hr (Fig. 3). Uptake by both varieties of embryo increased up to 0.29 M sucrose (Table 2). Higher concentrations did not greatly affect the rate of uptake. At the higher concentrations there was little difference in the rates of uptake by the two varieties, but embryos of round peas showed greater rates at the lower concentrations.

The relationship between uptake and metabolism of [^{14}C]sucrose is shown in Table 2. In these experiments the sum of the ^{14}C that was metabolized, present in the embryos as [^{14}C]sucrose, and recovered in the medium was determined for each sample, and compared with the amount of ^{14}C supplied. The sum of the former was 98 ± 1 (mean ± s.e.m. for 10 samples) of the latter. At each concentration, uptake of [^{14}C]sucrose exceeded the extent of its metabolism. At each concentration both the amount of [^{14}C]sucrose metabolized and the amount metabolized to insoluble material were higher for embryos of round peas than for those of wrinkled peas. For both varieties increase in the concentration of sucrose up to about 0.3 M led to increases in the amount metabolized, and more modest increases in the amount converted to insoluble products.

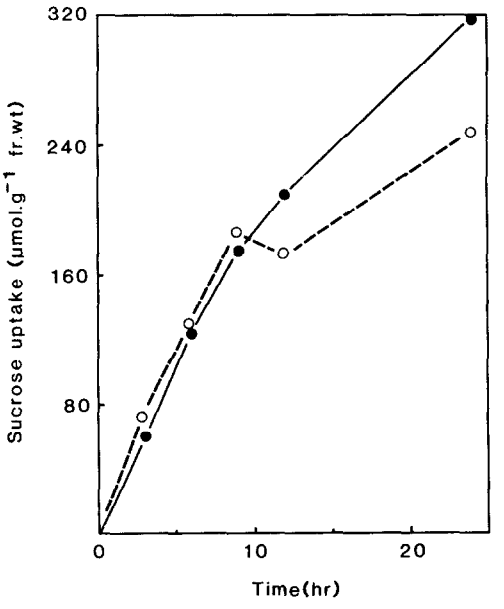

Fig. 3. Uptake of 0.53 M [U-^{14}C]sucrose by developing embryos of round (●) and wrinkled (○) peas.

Enzyme activities

For assay of acid invertase, homogenates of embryos were centrifuged at 11 000 *g* and both supernatant and sediment were assayed at five different pH values between pH 3 and 5. No activity could be detected in either fraction from either type of embryo. Alkaline invertase was found in the supernatant fractions (Fig. 4). We investigated whether this lack of acid invertase was due to its preferential inactivation during extraction of the embryos. These studies were done with samples that contained both varieties of embryo. For each test we prepared three samples, one of embryos, one of a comparable weight of a β stage club of the spadix of *Arum maculatum*, and one that was a mixture of similar weights of embryos and *Arum* club. The homogenates were centrifuged at 11 000 *g*. Acid invertase was measured in the sediment and the supernant: alkaline invertase was measured in the supernatant. For acid invertase, the activities in the mixed sample of embryo and *Arum* tissue were 77% (sediment) and 87% (supernatant) of those predicted from the measurements made on the separate components of the mixture. For alkaline invertase in the supernatant the corresponding value was 89%. A similar check was made for sucrose synthase and the value for the mixed sample was 101% of that predicted. The pH, and the

Table 2. Effect of concentration on uptake and metabolism of [U-^{14}C]sucrose by developing embryos of round and wrinkled peas

Concentration of [^{14}C]sucrose (M)	Uptake of [^{14}C]sucrose (μmol.g^{-1}fr. wt)		Total [^{14}C]sucrose metabolized (μmol.g^{-1}fr. wt)		[^{14}C]Sucrose converted to insoluble material (μmol.g^{-1}fr. wt)	
	Round	Wrinkled	Round	Wrinkled	Round	Wrinkled
0.06	42.5	18.7	18.0	4.1	15.3	2.2
0.18	77.5	63.4	22.6	8.6	17.2	4.3
0.29	113.0	94.4	24.3	10.7	18.6	6.1
0.41	109.7	96.6	19.6	13.1	13.9	3.7
0.53	95.6	100.8	15.6	11.1	9.2	4.6

Samples of six embryos were incubated in [^{14}C]sucrose for 6 hr and then divided into two sub-samples, each of three embryos, for analysis. Values are means of estimates, which were within 10% of each other, from the two sub-samples.

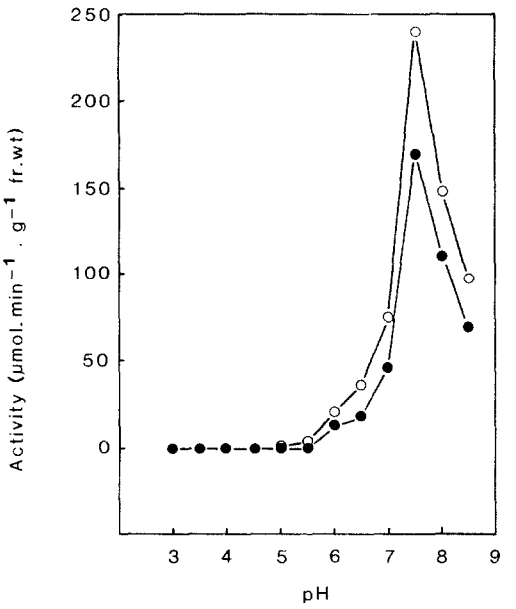

Fig. 4. Effect of pH on invertase activity in extracts of developing embryos of round (●) and wrinkled (○) peas. Values at pH 5.0 and below are for supernatant and pellet; values at pH 5.5 and above are for supernatant. The buffers used were the mixtures of 0.2 M Na_2HPO_4 and 0.1 M citric acid required to give the appropriate pH values.

concentration of each component, of the reaction mixtures for both alkaline invertase and sucrose synthase were optimized. Optimization of the pH for the assays of alkaline and acid invertase was done with extracts of samples of round embryos, and with extracts of wrinkled embryos. All other optimizations were carried out with extracts made from a mixed sample of round and wrinkled embryos. For both alkaline invertase and sucrose synthase we showed that activity was linearly related to time throughout the assay, and to the amount of extract assayed. Sucrose synthase was assayed with UDP, rather than any other nucleotide, because of the evidence that UDP is in the substrate *in vivo* [1].

We measured alkaline invertase and sucrose synthase during the development of both varieties of embryo (Table 3). In both the activities per embryo increased markedly throughout the period studied. However, only for sucrose synthase in wrinkled peas was there a significant increase in activity per g fresh weight. At all stages examined the activity of sucrose synthase was almost an order of magnitude greater than that of alkaline invertase. Finally, after the 100 mg stage, the activities of both enzymes were higher in embryos of wrinkled peas than in those of round peas.

DISCUSSION

The results of our feeding experiments were not seriously affected by losses during analysis or by microbial contamination. Our measurements of enzyme activities are sufficiently authenticated for us to argue that they reflect the maximum catalytic activities of the embryos.

The distribution of label from [^{14}C]sucrose shows that, in both varieties of embryo, storage material is the dominant product of sucrose metabolism. These results, and our measurements of starch accumulation (Fig. 2), show that the two varieties of embryo differ substantially in their immediate partitioning of incoming sucrose between storage products and respiration. This difference in partitioning appears as perhaps the major difference in

Table 3. Activities of sucrose synthase and alkaline invertase during development of embryos of round and wrinkled peas

Embryo fresh weight (mg)	Enzyme activity (nmol. min.$^{-1}$ per embryo)			
	Sucrose synthase		Alkaline invertase	
	Round	Wrinkled	Round	Wrinkled
100	165 ± 4	167 ± 20	17.6 ± 2.7	18.6 ± 1.5
200	217 ± 12	390 ± 18	30.1 ± 3.9	40.0 ± 1.2
300	388 ± 56	819 ± 29	47.0 ± 4.6	57.5 ± 2.3
400	629 ± 65	1280 ± 45	44.0 ± 2.5	63.6 ± 3.8

Values are means ± s.e.m. of estimates from at least four samples.

the metabolism of the two varieties of embryo. The extent to which this difference is responsible for the final starch and protein content of the mature seed is not apparent and is not likely to become so until we discover how the synthesis of the storage material is controlled.

Although we found other differences between the embryos of round and wrinkled seeds, none of them explains the above difference in sucrose partitioning. The differences in the rates of sucrose uptake are unlikely to be of prime significance because, at all concentrations of sucrose used, uptake significantly exceeded the amount metabolized. The concentration of sucrose available to the embryos in the normally developing pod is not known. Estimates for a related species, *Phaseolus vulgaris*, gave values of 0.1–0.2 M [6]. Thus the range of [U-^{14}C]sucrose concentrations that we used is likely to have included those that occur naturally. We suggest that the ability of the developing embryos to take up sucrose is unlikely to play a major role in the regulation of the metabolism of the developing embryos. The observation that embryos of round peas metabolized almost twice as much [^{14}C]sucrose as did the embryos of wrinkled peas may reflect a difference in the rates at which the two types of embryo metabolize sucrose. This is not necessarily so, as the incoming [^{14}C]sucrose may have been diluted to different extents in the two types of embryo. Support for this view is provided by the evidence that embryos of round peas contain 60 μmol sucrose per g fr. wt, whilst those of wrinkled peas contain 117 μmol per g fr. wt [7]. Against this dilution hypothesis is the fact that the difference in [^{14}C]sucrose metabolized by the two varieties was almost independent of [^{14}C]sucrose concentration up to 0.3 M, and the possibility that not all the sucrose in the embryo is in the cytosol. If the two varieties do differ in their rate of sucrose metabolism, then our enzyme measurements (Table 3) show that this could not be due to a greater capacity of embryos of round seeds to catalyse the initial breakdown of sucrose.

The absence of acid invertase, generally found in very high activities in rapidly growing plant tissues [8], is particularly striking. Whatever the role of this enzyme in other tissues, it seems clear that it is not responsible for significant breakdown of sucrose in developing embryos of pea. This leaves alkaline invertase and sucrose synthase as possible routes of sucrose breakdown. Both enzymes appear to be confined to the cytosol [9] and would thus appear to have immediate access to sucrose entering the cell. In an attempt to identify the relative roles of alkaline invertase and sucrose synthase, we compare their maximum catalytic activities at the 300 mg stage with estimates of the rates of sucrose breakdown in the embryos (Table 4). The maximum rate of sucrose uptake is taken as the upper estimate of the rate of sucrose metabolism. As the concentration of sucrose surrounding the embryos in their natural environment may well be lower than that required for maximum uptake, the rate of uptake at a lower concentration of sucrose is also given. Finally, we give our maximum estimate of the rate of [^{14}C]sucrose metabolism, but appreciate that dilution of the isotope with endogenous sucrose may make the value an appreciable underestimate of the rate of sucrose metabolism *in vivo*.

We make the following points from Table 4. Bearing in mind that alkaline invertase was assayed at 30° and all the other data were obtained at 25°, it is clear that the activity of alkaline invertase is close to or less than the estimates of the rate of sucrose breakdown *in vivo*. The maximum catalytic activities of sucrose synthase are well in excess of all estimates of the rate of sucrose breakdown. These results do not allow a definitive choice to be made between alkaline invertase and sucrose synthase as the means of immediate metabolism of the sucrose delivered to pea embryos. We suggest that both enzymes are likely to contribute to sucrose breakdown but that the contribution by sucrose synthase is likely to be appreciably greater than that by alkaline invertase. The essential difficulty with this proposal is the conversion of the UDP-glucose, formed by sucrose synthase, to hexose phosphates [1]. However, our previous demonstration that pea embryos contain significant amounts of pyrophosphate would allow conversion of this UDP-glucose to glucose 1-phosphate via UDP-glucose pyrophosphorylase.

EXPERIMENTAL

Materials. Substrates and cofactors were from Boehringer except that UDP-glucose and fructose 6-phosphate were from Sigma. [U-^{14}C]Sucrose was from the Radiochemical Centre, Amersham. Seeds of *Pisum sativum* L. cv. Birte (JI 1068 round-seeded) and cv. Greenshaft (JI 430 wrinkled-seeded) were generously supplied by the John Innes Institute, Norwich. Plants were grown in Fisons Levingtons compost at 15° at night and at a minimum of 18° by day in an 18-hr photoperiod of daylight supplemented with artificial light that gave a photon flux at soil level of 73 μmol. m^{-2}. sec^{-1}. Pods were tagged on the first day on which the flowers opened fully, and this was taken as the first day after anthesis.

Metabolism of [^{14}C]*sucrose.* Pods were harvested and sterilized by immersion in 7% (w/v) $NaClO_4$ for 10 min. All

Table 4. Comparison of activities of sucrose synthase and alkaline invertase with estimates of sucrose uptake and metabolism in developing embryos, at the 300 mg fr. wt stage, of round and wrinkled peas

	Value (μmol. min.$^{-1}$ g^{-1} fr. wt)	
Measurement	Round	Wrinkled
Sucrose synthase	0.97	2.68
Alkaline invertase	0.16	0.19
Maximum estimate of sucrose uptake	0.31	0.28
Sucrose uptake at 0.06 M	0.12	0.05
Maximum estimate of [^{14}C]sucrose metabolism	0.07	0.04

subsequent procedures up to the end of the chase, or pulse if there was no chase, were carried out under aseptic conditions. For the pulse and chase experiments (Table 1), nine embryos (each of fr. wt 250–350 mg) were suspended in 4 ml 'pulse medium' in an Erlenmeyer flask fitted with a centre-well that contained 10% (w/v) KOH to absorb $^{14}CO_2$. The pulse medium contained: 7 μM [U-^{14}C]sucrose (0.36 μCi.nmol^{-1}), 0.53 M mannitol, 67 mM asparagine, 24 mM homoserine, 29 mM serine, 23 mM glutamine, 21 mM alanine, 100 mM glycine plus the micronutrients and inorganic components of the medium described in ref. [10]. The flasks were incubated in the dark at 25° for 6 hr (pulse). Then the pulse medium was removed and the embryos were rinsed × 3 with, and resuspended in, chase medium and incubated for a further 18 hr (chase). The 'chase medium' was the same as the 'pulse medium' except that the [^{14}C]sucrose and mannitol were replaced with 0.53 M sucrose. At the end of the chase the embryos were divided into samples of three as described under results and each of these samples was analysed after killing by homogenizing in 2 vols $CHCl_3$–MeOH (2:1, v/v). The homogenate was centrifuged at 2400 *g* for 10 min and the supernatant was collected. This extraction was repeated and 0.2 vol. 0.04% (w/v) $CaCl_2$ was added to the combined supernatants. This mixture was shaken and then centrifuged at 2400 *g* for 10 min to give a lower phase that was extracted × 4 with 0.04% $CaCl_2$, evaporated to 2 ml at 15°, made up to 15 ml with 80% aq. EtOH, evaporated to 2 ml at 15°, made up to 5 ml with 80% aq. EtOH and counted to give ^{14}C in lipids. The material insoluble in $CHCl_3$–MeOH was extracted successively with boiling 80%, 20% aq. EtOH, H_2O, 40%, 20% EtOH, H_2O (twice), 80% EtOH (twice). These extracts plus the water-soluble material extracted in $CHCl_3$–MeOH were combined and reduced almost to dryness at 30° and made up to 25 ml with H_2O. Starch was removed by centrifugation and the supernatant, called the water-soluble material, was fractionated by ion-exchange and paper chromatography as in ref. [11]. Labelling of starch was determined by subjecting the starch obtained from the EtOH and H_2O extract, and the residue left after the above extraction, to the procedure described in ref. [12]. Labelling of protein was measured as in ref. [13].

To determine the time course of sucrose uptake (Fig. 3), samples of 12 embryos (each of fr. wt 250–350 mg) were incubated as above in 4 ml of 'chase medium' that contained 0.53 M [U-^{14}C]sucrose (2.4 nCi·μmol^{-1}). At each sampling time two embryos were removed, given 20 successive 30 sec rinses with 'chase medium' at 2°, plunged into liquid N_2, and then killed by homogenizing in 80% (v/v) aq. EtOH at 50°. We checked, as in ref. [14], that this rinsing effectively removed [^{14}C]sucrose from the free space. Total ^{14}C recovered in the homogenate plus any recovered as $^{14}CO_2$ during the incubation were summed and used as a measure of uptake.

For simultaneous measurement of [^{14}C]sucrose uptake and metabolism (Table 2) samples of six embryos (each of fr. wt 250–350 mg) were incubated in 4 ml of 'chase medium' to which [^{14}C]sucrose had been added and the concn of sucrose varied so that the final concn of [^{14}C]sucrose ranged from 0.06 M to 0.53 M, and the sp. act. from 6.1 to 0.7 nCi·μmol^{-1}. After a 6-hr incubation the embryos were divided into samples of three and then washed and killed as described earlier to give a measure of uptake. The homogenates were then fractionated as in the pulse and chase experiments to give total ^{14}C metabolized, except that the insoluble fraction was not subdivided into lipid, starch and protein but was kept as a single fraction.

Enzyme measurements. Samples of 1–3 embryos (each of fr. wt 100–400 mg) were homogenized in 5–10 ml extraction medium, first with a pestle and mortar and then with an all-glass homogenizer. We checked that this procedure left very few cells unbroken. For invertase the extraction medium was 19.45 mM Na_2HPO_4–0.28 mM citric acid, pH 8.0. The extract was dialysed against 5 l. 1.65 mM Na_2HPO_4–0.18 mM citric acid, pH 7.0 for assays at pH 6.0 or above, or 5 l. 0.77 mM Na_2HPO_4–0.62 mM citric acid, pH 4.0 for assays below pH 6.0. The assay, at 30°, was as in ref. [15] except that activity is given as glucose production, measured as in ref. [16]. The assay mixture for alkaline invertase contained, in 2 ml, 200 mM sucrose, 18.2 mM Na_2HPO_4–0.82 mM citric acid, pH 7.5. For sucrose synthase the extraction medium was 50 mM glycylglycine, pH 7.5. The homogenate was centrifuged at 100000 *g* for 30 min to give a supernatant that was dialysed against 5 l. 10 mM Tris–HCl, pH 8.0 for 3 hr and then assayed at 25° as in ref. [17] in a reaction mixture, 100 μl, that contained 50 μl extract, 33 mM Tris–HCl, pH 8.5, 13.3 mM UDP-glucose, 25 mM sucrose, 10 mM [U-^{14}C]fructose (8 Ci·m mol^{-1}). ^{14}C was measured by liquid scintillation counting. The scintillants were those described in either ref. [18] or [19]. ^{14}C in insoluble material was determined as in ref. [19]. Starch was assayed by measuring the glucose released by treatment with amyloglucosidase and α-amylase as described in ref. [16].

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