Apparent equilibrium constant and mass-action ratio for sucrose-phosphate synthase in seeds of *Pisum sativum*

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The aim of this work was to use preparations from germinating seeds of *Pisum sativum* to determine the apparent equilibrium constant of the reaction catalysed by sucrose-phosphate synthase (EC 2.4.1.14) and to compare this with the mass-action ratio of the reaction in the seeds. The apparent equilibrium constant ranged from 5.3 at 0.25 mM-MgCl₂, pH 7.0, to 62 at 10 mM-MgCl₂, pH 7.5. The sucrose phosphate content of the seeds, 23 nmol/g fresh wt., was determined by separating sucrose phosphate from sucrose by ion-exchange chromatography and then measuring the sucrose released by alkaline phosphatase. Comparison of equilibrium constants and mass-action ratios in the cotyledons of 38 h-germinated seeds showed that the reactions catalysed by glucose-6-phosphate isomerase, phosphoglucomutase and UDP-glucose pyrophosphorylase are close to equilibrium, and those catalysed by sucrose-phosphate synthase and sucrose phosphatase are considerably displaced from equilibrium *in vivo*.

INTRODUCTION

Our aim was to determine the apparent equilibrium constant (K_{app}) of the reaction catalysed by sucrose-phosphate synthase (EC 2.4.1.14) and then to compare K_{app} with mass-action ratios for the later steps in sucrose synthesis in the cotyledons of germinating peas. The apparent equilibrium constant was determined because the reaction catalysed by sucrose-phosphate synthase involves a proton. Sucrose-phosphate synthase and sucrose phosphatase (EC 3.1.3.24) catalyse the last two steps in sucrose synthesis and between them are likely to exercise appreciable control over flux. Evidence for the latter is provided by the observations that substantial concentrations of UDP-glucose and fructose 6-phosphate (Fru-6-P) are present in darkened leaves when sucrose synthesis is minimal (Gerhardt *et al.*, 1987), and that treatments that restrict sucrose synthesis can cause these compounds to accumulate (Stitt *et al.*, 1984). We do not know the relative contributions of sucrose-phosphate synthase and sucrose phosphatase to this control, in part because we do not know the extent to which the reaction catalysed by sucrose-phosphate synthase is displaced from equilibrium *in vivo*. This question may be answered by comparing K_{app} with the mass-action ratio (Newsholme & Start, 1973). Until now we have been unable to do this for sucrose-phosphate synthase because there is disagreement over K_{app} , and there is no authenticated measurement of the amount of sucrose phosphate in plant tissues.

For years the accepted value for K_{app} for the sucrose-phosphate synthase reaction has been 3250 at pH 7.5, an estimate made by Mendicino (1960) with a preparation from wheat germ. This was challenged by Barber (1985), who used the wheat-germ enzyme supplied by Sigma Chemical Co. and reported a value of 1.8. If the latter is correct, then the reaction may be very close to equilibrium *in vivo*, with the major control of this part of the pathway probably being exerted by sucrose phosphatase. Neither of the above estimates of K_{app} rest on direct measurements of all the reactants present in the final equilibrium mixtures, and both are vulnerable to contamination of the sucrose-phosphate synthase by glucose-6-phosphate isomerase. No tests for such contamination were reported by either Mendicino (1960) or Barber (1985). Mendicino (1960) supplied the enzyme with [¹⁴C]sucrose phosphate and UDP, and measured [¹⁴C]Fru-6-P

at equilibrium. From this the concentration of Fru-6-P was calculated and used in the calculation of the concentrations of the other reactants. Contamination by glucose-6-phosphate (Glc-6-P) isomerase would convert some of the [¹⁴C]Fru-6-P into [¹⁴C]Glc-6-P, and thus the former would not be a true measurement of the total [¹⁴C]Fru-6-P formed. Barber (1985) incubated enzyme with [¹⁴C]UDP-glucose and Fru-6-P and determined the labelling of [¹⁴C]sucrose phosphate at equilibrium. The concentration of Fru-6-P at equilibrium was then calculated by subtracting the amount of sucrose phosphate formed from the initial concentration of Fru-6-P. Again, any conversion of Fru-6-P into Glc-6-P would introduce errors. We have obtained a preparation of sucrose-phosphate synthase from germinating pea seeds that is sufficiently free from glucose-6-phosphate isomerase and other contaminants to permit the determination of K_{app} by direct measurement of all four reactants (Lunn & ap Rees, 1990). The same preparation also enabled us to make enough sucrose phosphate to develop and authenticate an assay for this compound and thus determine the mass-action ratio for sucrose-phosphate synthase in pea seeds and to compare this with those of the other steps in the pathway.

MATERIALS AND METHODS

Materials

Seeds of pea (*Pisum sativum* L. var. Kelvedon Wonder) were germinated by washing in running tap water for 38 h and were used at once. The testa and embryo were removed, and each sample consisted of a single cotyledon.

Substrates and cofactors were from Sigma Chemical Co. (Poole, Dorset, U.K.), except that glucose 1-phosphate (Glc-1-P), ATP, NADP⁺, NAD⁺ and NADH were from Boehringer Corp. (Lewes, Sussex, U.K.) and [U-¹⁴C]Fru-6-P was from Amersham International (Amersham, Bucks., U.K.). [¹⁴C]Sucrose 6'-phosphate was made by incubating at 25 °C for 18 h, in 1.0 ml, 10 mM-[¹⁴C]Fru-6-P (3.87 GBq/mol), 30 mM-UDP-glucose, 50 mM-Hepes/NaOH (pH 7.5), 10 mM-MgCl₂ and 0.1 unit of sucrose-phosphate synthase purified as described by Lunn & ap Rees (1989). At the end of the incubation the reaction mixture was kept at 100 °C for 2 min, centrifuged at 13 000 g for

Abbreviations used: Fru-6-P, fructose 6-phosphate; Glc-1-P, glucose 1-phosphate; Glc-6-P, glucose 6-phosphate.

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2 min, and the supernatant was chromatographed on Whatman no. 3MM paper with propan-1-ol/conc. NH_3 (sp.gr. 0.880)/water (6:3:1, by vol.). Sucrose phosphate was located by spraying reference tracks with 75 mM-ammonium molybdate in 1.5 M-HCl/4 M- NH_4Cl (Dawson *et al.*, 1986), eluted and concentrated *in vacuo* at 25 °C. Incubation of the isolated sucrose phosphate overnight with 1 unit of alkaline phosphatase/ml yielded equimolar amounts of P_i and sucrose. Treatment of the sucrose with invertase gave equimolar amounts of fructose and glucose. Further analysis showed that the sucrose phosphate preparation contained no detectable sucrose, fructose, glucose or UDP, and only 0.3% UDP-glucose, 0.7% Fru-6-P, 1.4% Glc-1-P and 0.3% Glc-6-P with respect to sucrose phosphate.

Enzymes were from Boehringer Corp., except that pyrophosphate:fructose-6-phosphate 1-phosphotransferase (EC 2.7.1.90, from *Propionibacterium freudenreichii*) and sucrose-phosphate synthase were from Sigma Chemical Co. The sucrose-phosphate synthase used in the measurement of K_{app} and in the synthesis of sucrose phosphate was prepared and checked for contaminants as described by Lunn & ap Rees (1990).

Extraction of metabolites

Samples of single cotyledons were freeze-clamped (ap Rees *et al.*, 1977) within 2 min of removal from the seed, dropped into liquid N_2 in a mortar and ground to a powder under liquid N_2 . Subsequent procedure varied with the metabolite. Except for sucrose phosphate and PP_i , the frozen powdered tissue was poured in liquid N_2 into a 50 ml centrifuge tube, and 1 ml of ice-cold 0.6 M- HClO_4 was added just before the liquid N_2 evaporated. The resulting suspension was vortex-mixed until the tissue began to thaw, then left in ice for 15 min and centrifuged at 2 °C for 2 min at 30000 g. The sediment was washed ($2 \times 250 \mu\text{l}$ of water), and washings and supernatant were combined, adjusted to pH 7–7.5 with 5 M-KOH/1 M-triethanolamine, left in ice for 5 min and centrifuged at 40000 g for 2 min at 2 °C. The sediment was washed twice ($2 \times 250 \mu\text{l}$ of water), and washings and supernatant were combined and assayed. For sucrose phosphate the procedure was similar, except that 3.1 M- HClO_4 was used to kill the tissue and the sample was left in ice in the HClO_4 for 30 min. The final extract was passed through a column (70 mm $\times$ 10 mm) of Dowex 1 $\times$ 8-200 anion-exchange resin that had previously been treated with, successively, 25 ml of 2 M-NaOH, 50 ml of water, 100 ml of 8 M-formic acid, 100 ml of water, 150 ml of 0.1 M-sodium acetate and 50 ml of 4 M-acetic acid. This exhaustive washing was necessary to remove material that otherwise interfered with the subsequent assay of sucrose. After the addition of the extract, the column was washed with 60 ml of water and then eluted with 50 ml of 4 M-formic acid; the eluate was evaporated to dryness at 30 °C *in vacuo* and the residue was dissolved in 5 ml of 50 mM-Pipes/NaOH/5 mM- MgCl_2 at pH 7.0. For PP_i , the powdered tissue was killed and extracted with trichloroacetic acid as described by Weiner *et al.* (1987).

Assay of metabolites

Hexose monophosphates and UDP-glucose were assayed, as described by Stitt *et al.* (1980), in the same reaction mixture, that contained, in 660 μl , 83 mM-Tris/HCl, pH 8.1, 4 mM- MgCl_2 and 12.5 mM-NADP $^+$. Glc-6-P, Fru-6-P and Glc-1-P were assayed by the sequential addition of 0.28 unit of glucose-6-phosphate dehydrogenase (EC 1.1.1.49), 0.7 unit of glucose-6-phosphate isomerase (EC 5.3.1.9) and 0.4 unit of phosphoglucomutase (EC 5.4.2.2). Then UDP-glucose was assayed by adding 5 μl of 15 mM- $\text{Na}_2\text{H}_2\text{P}_2\text{O}_7$ and 0.2 unit of UDP-glucose pyrophosphorylase (EC 2.7.7.9). PP_i was measured by a modification of the method of Weiner *et al.* (1987) in a reaction mixture

(700 μl) of 50 mM-Tris/HCl, pH 7.5, 2 mM- MgCl_2 , 17 μM -NADH, 1 mM-Fru-6-P, 0.3 unit of aldolase (EC 4.1.2.13), 3.5 units of triose-phosphate isomerase (EC 5.3.1.1) and 1.23 units of glycerol-3-phosphate dehydrogenase (EC 1.1.1.8). The reaction was started by addition of 0.5 unit of pyrophosphate:fructose-6-phosphate 1-phosphotransferase. For each of the above assays we demonstrated the precise stoichiometry expected.

For analysis of reaction mixtures of sucrose-phosphate synthase, UDP was measured by coupling to NADH oxidation via pyruvate kinase and lactate dehydrogenase (Lunn & ap Rees, 1990). Uridine nucleotides in tissue extracts were measured as described by Keppler *et al.* (1985). Complications arose from contamination of the commercial preparations of UDP-glucose dehydrogenase (EC 1.1.1.22) with both UDP-glucose pyrophosphorylase and nucleoside-diphosphate kinase (EC 2.7.4.6). To avoid these, we first measured the sum of UTP+UDP, and then measured UDP, and obtained UTP by difference. The first reaction mixture (920 μl) contained 272 mM-glycine/KOH, pH 8.7, 1.6 mM-NAD $^+$, 4.3 mM-EDTA and 0.06 unit of UDP-glucose dehydrogenase. When absorbance became constant we added 40 μl of a mixture that contained 49 mM-Glc-1-P, 464 mM-magnesium acetate, 0.9 unit of UDP-glucose pyrophosphorylase, 96 mM-ATP and 1 unit of nucleoside-diphosphate kinase. Withholding Mg^{2+} until all the UDP-glucose had been removed prevented the contaminants of the dehydrogenase from reacting with UDP and UTP until the remaining coupling enzymes were added. UDP alone was measured by incubating extract in 300 μl of 93.3 mM-triethanolamine/KOH, pH 7.5, 1.3 mM-magnesium acetate, 8.3 mM-Glc-1-P and 3 units of UDP-glucose pyrophosphorylase for 30 min at 25 °C to convert UTP into UDP-glucose. Then 500 μl of 0.5 M-glycine/KOH (pH 8.7)/3 mM-NAD $^+$ /8 mM-EDTA, containing 0.06 unit of UDP-glucose dehydrogenase, was added to remove the UDP-glucose. When this was complete, UDP was measured by adding 20 μl of 96 mM-ATP/470 mM-magnesium acetate containing 1 unit of nucleoside-diphosphate kinase. All measurements were corrected for any possible contamination of the coupling enzymes with uridine nucleotides by running blanks with water instead of extract. Assay of commercial UDP and UTP showed decreases of 1.8 ± 0.7 and 1.6 ± 0.2 (means $\pm$ S.D. for 5 and 4 samples respectively) mol of NAD $^+$ /mol of nucleotide respectively. Thus the assays underestimated UDP and UTP by 10 and 20% respectively.

Sucrose phosphate was assayed by separating it from sucrose by ion-exchange chromatography, treating it with phosphatase and measuring the sucrose released. Samples (2.5 ml) of the material eluted from the ion-exchange column and dissolved in 50 mM-Pipes/NaOH (pH 7.0)/5 mM- MgCl_2 were incubated with 2.5 units of alkaline phosphatase (EC 3.1.3.1) for 12 h at 37 °C. Then samples (1 ml) were assayed for sucrose (Outlaw & Tarczynski, 1984) in a reaction mixture (1.03 ml) containing 1 mM-ATP, 0.4 mM-NADP $^+$, 0.3 unit of hexokinase (EC 2.7.1.1), 0.15 unit of glucose-6-phosphate dehydrogenase and 25 units of invertase (EC 3.2.1.26). The invertase had been dialysed overnight against 1000 vol. of 0.32 M-citric acid/NaOH, pH 4.6, at 4 °C. Duplicate assays were carried out in which 0.33 unit of glucose-6-phosphate isomerase was included to measure fructose as well as glucose. For all values reported we showed that NADP $^+$ reduction in the presence of the isomerase was double that in its absence. Control assays were made on extracts that had not been treated with alkaline phosphatase; no sucrose was detected. We also showed that no sucrose could be detected in the preparation of alkaline phosphatase that we added. Further, no sucrose was detected when alkaline phosphatase (5 units) was incubated overnight at 37 °C in a mixture (5 ml) of 125 nmol of Glc-6-P, 31 nmol of Fru-6-P, 9 nmol of Glc-1-P, 62 nmol of

UDP-glucose and 100 nmol of fructose in 50 mM-Pipes/NaOH (pH 7.0)/5 mM-MgCl₂. Thus the alkaline phosphatase was not significantly contaminated by sucrose-phosphate synthase, sucrose synthase, sucrose phosphorylase, or any other enzyme capable of catalysing the synthesis of sucrose from the hexose and hexose phosphates present in the extracts.

Determination of K_{app}

The initial reaction mixture contained, in 200 μ l, 0.1 unit of purified sucrose-phosphate synthase (Lunn & ap Rees, 1990), 5 mM-UDP-glucose, 5 mM-Fru-6-P in 50 mM-Pipes/NaOH, pH 7.0 or 7.5, and 0.25 mM- or 10 mM-MgCl₂, and was incubated at 25 °C for 16 h before being held for 2 min in a water bath at 100 °C. Samples (up to 10 μ l) were then assayed for all four reactants.

RESULTS AND DISCUSSION

Apparent equilibrium constant (K_{app})

The properties of the preparation of sucrose-phosphate synthase used to determine K_{app} have been described (Lunn & ap Rees, 1990). Specific activity was 4.2 μ mol/min per mg of protein, and there was no significant activity, under the conditions used to determine K_{app} , of invertase, sucrose synthase, glucose-6-phosphate isomerase, or any other enzyme capable of metabolizing Fru-6-P, UDP-glucose, sucrose phosphate or UDP. Both pH and Mg²⁺ concentration will affect K_{app} of sucrose-phosphate synthase. We chose pH 7.0 and 7.5 and 10 mM- and 0.25 mM-MgCl₂ as values that would span the range of pH and Mg²⁺ concentrations in the cytosol, where sucrose-phosphate synthase is located. Measurements of UDP showed that under optimum conditions the reaction reached equilibrium in 1 h. As our chosen conditions were slightly sub-optimal, we allowed the reaction to proceed for 16 h before inactivating the enzyme and measuring the amounts of reactants present. Evidence that equilibrium was reached was obtained by adding a further 0.1 unit of sucrose-phosphate synthase to 100 μ l of the equilibrium mixture, incubating at 25 °C for 1 h and assaying UDP. The second incubation did not alter the amount of UDP detected. We checked that boiling, the method used to stop the reaction, did not alter the amounts of the reactants in the equilibrium mixture. We did this by comparing boiling with treatment with HClO₄ as a means of stopping the reaction. We found that the amounts of reactants were the same in untreated mixtures analysed directly and in those where the enzyme was inactivated by boiling or by addition of 1 vol. of 1.4 M-HClO₄. We also showed that the expected amounts of reactants were present at the start of the reaction. We did this by measuring each reactant in a reaction mixture to which boiled enzyme had been added.

Table 1 shows the composition of the reaction mixtures at equilibrium. There was very close agreement between the results obtained with two entirely separate preparations of sucrose-phosphate synthase. The values for the stoichiometry of the reaction were very close to those expected. The estimates of Fru-6-P consumed are slightly greater than predicted. This was probably due to the long incubation time and very slight activity of glucose-6-phosphate isomerase. The difference is too small to have a significant effect on the estimation of K_{app} . Our values for the latter range from 5 to 65 according to pH and concentration of Mg²⁺. Increasing the concentration of Mg²⁺ from 0.25 mM to 10 mM resulted in a 5-fold increase in K_{app} . This suggests that Mg²⁺ does not react stoichiometrically, but is involved in a separate reaction to form a complex with UDP. The cytosolic pH of plant cells is close to pH 7.3 (Roberts *et al.*, 1984; Yazaki *et al.*, 1988), and the concentration of free Mg²⁺ has been

Table 1. Contents of equilibrium reaction mixtures of sucrose-phosphate synthase

Initial reaction mixtures contained 1 μ mol of Fru-6-P, 1 μ mol of UDP-glucose and 0.1 unit of sucrose-phosphate synthase in 200 μ l of 50 mM-Pipes/NaOH, pH 7.0 or 7.5, with MgCl₂ at 0.25 or 10 mM. After incubation at 25 °C for 16 h, the reaction mixtures were placed at 100 °C for 2 min, cooled and assayed for Fru-6-P, UDP-glucose, UDP and sucrose phosphate. The results are presented for experiments with two separate preparations of sucrose-phosphate synthase.

pH	[MgCl ₂] (mM)	Metabolite content (nmol/reaction mixture)				K_{app}
		Fru-6-P	UDP-glucose	UDP	Sucrose phosphate	
7.0	0.25	315	296	688	727	5.4
		289	302	669	673	5.2
7.0	10	161	180	849	881	25.8
		167	148	830	805	27.0
7.5	0.25	219	251	720	811	10.6
		219	232	746	662	9.7
7.5	10	129	103	881	894	59.3
		116	96	878	822	64.8

Table 2. Conversion of fructose 6-phosphate into glucose 6-phosphate by commercial preparations of sucrose-phosphate synthase from Sigma Chemical Co.

Reaction mixtures were set up and incubated as described in Table 1, except that 0.1 unit of sucrose-phosphate synthase from wheat germ as supplied by Sigma was used. At the end of the incubation, samples of the boiled reaction mixture were assayed for Glc-6-P and Fru-6-P. The initial reaction mixtures contained 30 nmol of Glc-6-P, which we found to be present as a contaminant of the commercial preparation of Fru-6-P.

pH	[MgCl ₂] (mM)	Metabolite content (nmol/ reaction mixture)	
		Glc-6-P	Fru-6-P
7.0	0.25	540	154
7.0	10	469	129
7.5	0.25	444	129
7.5	10	476	141

estimated as 0.4 mM (Yazaki *et al.*, 1988). Thus *in vivo* K_{app} for the sucrose-phosphate synthase reaction is probably close to 10.

Our estimate of K_{app} differs from the published values. We suggest that ours is the more reliable estimate because it is based on measurements of all four reactants and does not depend on assumptions that are undermined by the presence of significant activity of glucose-6-phosphate isomerase. The high value reported by Mendicino (1960) may be questioned on theoretical grounds, as it implies that the free energy of hydrolysis of the glycosidic bond of sucrose 6'-phosphate is very much smaller than that of sucrose (Neufeld & Hassid, 1963). This, our own estimates and the fact that neither the sucrose-phosphate synthase nor the sucrose phosphate used by Mendicino (1960) were shown to be uncontaminated lead us to reject Mendicino's value. The difference between our estimate and that of Barber (1985) is almost certainly due to contamination of the Sigma

Table 3. Tissue contents of metabolites involved in sucrose synthesis in cotyledons of 38 h-germinated seeds of pea

Samples of single cotyledons were freeze-clamped and then killed and extracted with 0.6 M-HClO₄, except that 3.1 M-HClO₄ and 0.98 M-trichloroacetic acid were used for sucrose 6'-phosphate and PP_i respectively. In each recovery experiment the two cotyledons from a single seed were taken as duplicate samples; one was freeze-clamped, killed, and extracted as above, and the other was treated similarly except that measured amounts of metabolites were added to the freeze-clamped tissue. The difference in metabolite content between the two samples was used to estimate the recovery of the added metabolite. Data are means $\pm$ S.D. from the numbers of cotyledons shown in parentheses.

Metabolite	Tissue content (nmol/g fresh wt.)	Recovery	
		Amount added (nmol)	Percentage recovered
Glc-6- <i>P</i>	479 $\pm$ 108 (10)	149–198	99 $\pm$ 9 (10)
Fru-6- <i>P</i>	120 $\pm$ 27 (10)	28–52	94 $\pm$ 15 (10)
Glc-1- <i>P</i>	35 $\pm$ 10 (10)	14–22	91 $\pm$ 18 (10)
UDP-glucose	238 $\pm$ 53 (10)	47–95	97 $\pm$ 16 (10)
UTP	67 $\pm$ 21 (6)	13–25	95 $\pm$ 19 (6)
UDP	32 $\pm$ 5 (6)	7–13	98 $\pm$ 25 (6)
PP _i	7 $\pm$ 1 (6)	2	93 $\pm$ 17 (6)
Sucrose 6'-phosphate	23 $\pm$ 3 (7)	4–6	101 $\pm$ 14 (7)

Table 4. Comparison of mass-action ratios, apparent equilibrium constants and free-energy changes of reactions of sucrose synthesis in cotyledons of 38 h-germinated seeds of pea

Mass-action ratios are calculated from the data in Table 3. Where possible, ratios were determined for each sample and are given as means $\pm$ S.D. for the numbers of samples measured (shown in parentheses). As PP_i and sucrose 6'-phosphate were measured on different samples, average values for each were determined and then used to calculate the ratios for each sample in which the other metabolites were measured. *K'* values are from the following references: glucose-6-phosphate isomerase (Dyson & Noltman, 1968), phosphoglucomutase (Lehninger, 1982), UDP-glucose pyrophosphorylase (Weiner *et al.*, 1987), sucrose-phosphate synthase (Table 1). Standard free-energy change is calculated from $\Delta G^0 = -RT \cdot \ln K'$. Free-energy change *in vivo* is calculated from $\Delta G = \Delta G^0 + RT \cdot \ln I$, where *I* is mass-action ratio, *R* is 8.314 J $\cdot$ mol⁻¹ $\cdot$ K⁻¹ and *T* is 298 K.

Enzyme	Mass-action ratio	K'	$\Delta G^{0'}$ (kJ · mol ⁻¹)	ΔG <i>in vivo</i> (kJ · mol ⁻¹)	
Phosphoglucose isomerase	$\frac{[\text{Glc-6-}P]}{[\text{Fru-6-}P]}$	4.000 ± 0.33 (10)	3.70	− 3.2	+ 0.2
Phosphoglucomutase	$\frac{[\text{Glc-1-}P]}{[\text{Glc-6-}P]}$	0.074 ± 0.018 (10)	0.05	+ 7.3	+ 0.9
UDP-glucose pyrophosphorylase	$\frac{[\text{UDP-glucose}][\text{PP}_i]}{[\text{Glc-1-}P][\text{UTP}]}$	0.990 ± 0.36 (7)	0.31	+ 2.9	+ 2.9
Sucrose-phosphate synthase	$\frac{[\text{Sucrose phosphate}][\text{UDP}]}{[\text{UDP-glucose}][\text{Fru-6-}P]}$	0.029 ± 0.01 (6)	10.00	− 5.7	− 14.5

sucrose-phosphate synthase, used by Barber, by glucose-6-phosphate isomerase. If we used the Sigma enzyme, instead of our own preparation, we found massive conversion of Fru-6-*P* into Glc-6-*P* (Table 2). This was true for each pH and each concentration of Mg²⁺. At equilibrium the ratios of Glc-6-*P* to Fru-6-*P* were 3.4–3.6 (Table 2). This is sufficiently close to *K_{app.}* for the glucose-6-phosphate isomerase reaction, 3.7 at 25 °C (Dyson & Noltman, 1968), to make it clear that the Sigma preparation is appreciably contaminated by the isomerase. Such contamination would lead to underestimation by Barber's (1985) method.

Values of 1.4–6.7 for *K_{app.}* for the reaction catalysed by sucrose synthase (EC 2.4.1.13) in the direction of sucrose synthesis have been reported (Pontis, 1977). The closeness of these estimates to ours for sucrose-phosphate synthase suggests that the free energy of hydrolysis of the glycosidic bonds in sucrose and sucrose phosphate is not very different.

Mass-action ratio for sucrose-phosphate synthase

We compared *K_{app.}* and the mass-action ratio for sucrose-

phosphate synthase, and did the same for other enzymes involved in sucrose synthesis. The mass-action ratios depend upon reliable estimates of the amounts of substrates and products in the intact tissue. For each metabolite assayed we completed recovery experiments to check for losses during extraction and analysis. For each test we used the two cotyledons from a single seed as duplicate samples. One was freeze-clamped and extracted in the usual way; the other was treated similarly, except that measured amounts of the metabolites were added to the freeze-clamped tissue before addition of the HClO₄ or trichloroacetic acid. The difference in the amounts found in the two extracts is used to estimate the recovery of the added compound. The amounts added were comparable with the quantities normally extracted from the tissue sample. Recoveries are satisfactory (Table 3). As the assay for sucrose phosphate is new, we made a further check for this compound by adding 4–6 nmol of [*fructosyl*-¹⁴C]sucrose phosphate (3.91 GBq/mol) to freeze-clamped samples. Analysis of the distribution of this label showed that 92 % of that added was recovered and that 99 % of this was in the acidic fraction

that was subsequently treated with alkaline phosphatase. We suggest that our measurements of metabolites are reliable.

We have used the data in Table 3 to calculate the mass-action ratios and free-energy changes of the reactions involved in sucrose synthesis in the cotyledons of germinating peas (Table 4). We argue that our use of overall contents of metabolites to calculate these ratios does not introduce significant error into our conclusions. For the compounds in question, the major source of error is that they may not be confined to the cytosol, the site of sucrose synthesis, as a fraction may be in the plastid. This fraction is likely to be small in germinating peas. First, it is highly probable that there were very few intact plastids in the germinating cotyledons, as there is good evidence that the plastid envelope ruptures during the maturation of the seed (Bain & Mercer, 1966). Second, in such a starch-filled seed the total volume of the stroma of the plastid will be very small relative to that of the cytosol. Finally, there is good evidence that key substrates and their enzymes are confined to the cytosol, namely UDP-glucose, PP_i, UDP-glucose pyrophosphorylase and sucrose-phosphate synthase (Stitt *et al.*, 1987; ap Rees, 1987).

Inspection of Table 4 strongly suggests that the reactions catalysed by glucose-6-phosphate isomerase, phosphoglucose-mutase and UDP-glucose pyrophosphorylase are very close to equilibrium in germinating peas, which are thus similar to the two other plant tissues examined in this respect, spinach leaves (Stitt *et al.*, 1987) and pea embryos (Edwards & ap Rees, 1986). In contrast, the sucrose-phosphate synthase step is appreciably displaced from equilibrium and must be regarded as being essentially irreversible *in vivo*. The equilibrium position of the sucrose phosphatase reaction *in vivo* can only be assessed indirectly, because we lack techniques to measure the amounts of sucrose and P_i in the cytosol of the non-photosynthetic cells of plants. Values of 40 mM-sucrose and 5 mM-P_i are available for spinach leaf (Stitt *et al.*, 1987). If we assume comparable values and a cytosolic volume of 100 µl/g fresh wt. for pea seeds, then the mass-action ratio for sucrose phosphatase, from the above values and those in Table 3, is calculated as 0.016. We have estimated K_{app} for the sucrose phosphatase reaction as follows. The standard free-energy change at pH 7.0 for the sucrose phosphatase step was obtained by summing the values available for the forward reactions catalysed by sucrose synthase and fructose 6-phosphatase, and the reverse reaction catalysed by sucrose-phosphate synthase: -4.7 (Pontis, 1977), -15.9 (Lehninger, 1982), +4.1 (Table 1) kJ/mol respectively. A value of -16.5 kJ/mol is obtained. This gives a value of 780 for K_{app} for the sucrose phosphatase reaction. This value is so far from 0.016,

our estimate of the mass-action ratio, that it is clear that the sucrose phosphatase reaction is considerably displaced from equilibrium in pea seeds. Our evidence that the reactions catalysed by sucrose-phosphate synthase and by sucrose phosphatase are appreciably displaced from equilibrium *in vivo* strongly suggest that both enzymes make significant contributions to the control of sucrose synthesis, at least in the cotyledons of germinating peas.

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