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PHOSPHOENOLPYRUVATE CARBOXYKINASE AND GLUCONEOGENESIS IN COTYLEDONS OF *CUCURBITA PEPO*

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Summary

1. The aim of this work was to investigate the role of phosphoenolpyruvate carboxykinase (ATP:oxaloacetate carboxy-lyase (transphosphorylating) EC 4.1.1.49) in the conversion of fat to sugar by the cotyledons of seedlings of *Cucurbita pepo*.

2. The enzyme was partially purified from the cotyledons of 5-day-old seedlings. The Michaelis constants for oxaloacetate and ATP were 56 and 119 μM, respectively. The decarboxylation reaction was optimum at pH 7.4. A range of intermediary metabolites did not affect the activity of the enzyme, but 3-mercaptopicolinic acid at μM concentrations was an effective inhibitor.

3. Centrifugation of extracts of 5-day-old cotyledons sedimented appreciable proportions of the ribulosebisphosphate carboxylase, isocitrate lyase and fumarate hydratase present but very little of the phosphoenolpyruvate carboxykinase.

4. Measurements of phosphoenolpyruvate carboxykinase of cotyledons during germination showed that the maximum catalytic activity exceeded, and changed coincidently with, the rate of gluconeogenesis.

5. 3-Mercaptopicolinic acid inhibited gluconeogenesis from [1-^{14}C]- and [2-^{14}C]acetate supplied to excised cotyledons. The detailed distribution of ^{14}C indicated inhibition of the conversion of oxaloacetate to phosphoenolpyruvate.

6. It is concluded that in marrow cotyledons phosphoenolpyruvate carboxykinase is in the soluble phase of the cytoplasm and catalyses a component reaction of gluconeogenesis.

Introduction

It is generally accepted that the conversion of oxaloacetate to phosphoenolpyruvate in gluconeogenesis during the germination of fatty seedlings is catalysed by phosphoenolpyruvate carboxykinase (ATP:oxaloacetate carboxy-lyase

(transphosphorylating), EC 4.1.1.49) [1,2]. However, this enzyme has not been isolated from gluconeogenic tissues of higher plants, and there is disagreement as to whether such tissues contain sufficient of the enzyme to mediate the observed rates of gluconeogenesis. Cooper and Beevers [3] report an activity of the enzyme for castor bean endosperm that is consistent with its proposed role in gluconeogenesis. In contrast, in a subsequent paper, Benedict [4] reports an activity for the same tissue that is approx. 0.1% of the observed rate of gluconeogenesis. The assay used in the latter work was the one employed in the study that provides nearly all the evidence that extracts of gluconeogenic tissues of plants contain phosphoenolpyruvate carboxykinase [2]. The aim of the work reported in the present paper was to investigate the role of this enzyme in gluconeogenesis in the cotyledons of marrow seedlings.

Materials and Methods

Materials. 3-Mercaptopicolinic acid was given to us by Dr. H.L. Saunders, Smith Kline and French Laboratories, Philadelphia, Pa. [^{14}C]Acetate was obtained from the Radiochemical Centre, Amersham; substrates, cofactors and enzymes were from Boehringer, Mannheim, except for ribulosebisphosphate which came from the Sigma Chemical Co.

Marrows (*Cucurbita pepo* L. var. medullosa Alef.) were grown and harvested as described previously [5] except that growth was in continuous light. Unless we state otherwise all experiments were done with cotyledons from 5-day-old seedlings.

Preparation of extracts. All extractions were made at 4°C and the extracts were kept at 1°C until assayed. For the partial purification of phosphoenolpyruvate carboxykinase we used a pestle and mortar to homogenize 25—35 g fresh weight of cotyledons in 5 vols. 25 mM potassium phosphate buffer (pH 6.7) that contained 10 mM EDTA and 10 mM GSH. The homogenate was centrifuged at 105 000 $\times g$ for 30 min and the supernatant was used as the source of enzyme. For measurement of maximum catalytic activities, 2 g fresh weight of cotyledons were homogenized in 10 ml 40 mM glycylglycine buffer (pH 6.7) that contained 10 mM EDTA and 10 mM dithiothreitol. Complete cell breakage was achieved as described previously [5]. The homogenate was centrifuged as above and the supernatant was assayed at once. For cell fractionation, 10 g fresh weight of cotyledons were gently extracted with 10 ml 0.167 M glycylglycine buffer (pH 7.4) which contained 0.4 M sucrose and 0.5 mM dithiothreitol. The procedure was as described previously for the preparation of proplastids [6]. The extract was filtered through two and then eight layers of muslin, and then centrifuged at 2500 $\times g$ for 15 min. The sediment was re-suspended in 2.0 ml 0.167 M glycylglycine buffer (pH 7.4); the supernatant was centrifuged at 10 800 $\times g$ for 15 min to give a sediment, that was re-suspended in 1 ml 0.167 M glycylglycine buffer (pH 7.4), and a final supernatant.

Partial purification of phosphoenolpyruvate carboxykinase. The following was carried out at 4°C. The supernatant from the crude homogenate was treated with 1 vol. $(NH_4)_2SO_4$, saturated at 4°C (pH 6.7). The mixture was stirred for 15 min and then centrifuged at 15 000 $\times g$ for 10 min. The precipitate was dissolved in 20 ml 25 mM potassium phosphate buffer, pH 6.7, which

contained 0.5 mM EDTA and 1.0 mM GSH (buffer A) and then dialysed against the same buffer for 20 h. The dialysis residue was absorbed onto 4 g calcium phosphate gel, prepared as described by Davies and Davies [7]. The gel was suspended in the dialysis residue for 10 min and the suspension centrifuged at 7000 $\times g$ for 10 min. The supernatant was discarded and the gel was re-suspended in 0.5 M KCl dissolved in buffer A. After 10 min the suspension was centrifuged at 7000 $\times g$ for 5 min. The supernatant was retained, and the gel was eluted in the same way once more. Both eluates were combined and treated with 1 vol. $(NH_4)_2SO_4$ saturated at 4°C (pH 6.7). After 10 min the precipitate was collected by centrifuging at 15 000 $\times g$ for 10 min and dissolved in buffer A. The resulting solution was dialysed against buffer A for 20 h. The dialysis residue was then applied to a column (2.5 × 25 cm) of DEAE-Sephadex A-50 equilibrated with buffer A. The enzyme was eluted with a 50 ml linear gradient of KCl (0—0.2 M) dissolved in buffer A. Fractions of 5.0 ml were collected and the one with the highest specific activity of phosphoenolpyruvate carboxykinase and the lowest activity of malate dehydrogenase was applied to a column (2.5 × 25 cm) of Sephadex G-200, equilibrated with buffer A that contained 0.15 M KCl. The latter solution was used to elute the enzyme, and fractions of 5 ml were collected. Those with the highest specific activity were used at once for characterization of the enzyme.

Enzyme assays. All enzymes were measured at 25°C in 3.0-ml reaction mixtures. The carboxylation reaction of phosphoenolpyruvate carboxykinase was measured according to Cooper et al. [8]: the reaction mixture contained 2 mM phosphoenolpyruvate, 2 mM ADP, 1 mM $MnCl_2$, 50 mM $KHCO_3$, 0.35 mM NADH, 4.2 mM GSH and 6 units malate dehydrogenase in 40 mM 2-(*N*-morpholino)-ethane sulfonic acid buffer, pH 6.7. We used this assay to measure the enzyme in crude extracts. Under the conditions of the assay such extracts showed no activity of lactate dehydrogenase. The decarboxylation reaction of phosphoenolpyruvate carboxykinase was measured according to Chang and Lane [9]: the reaction mixture contained 0.5 mM oxaloacetate, 2 mM ATP, 1 mM $MnCl_2$, 0.35 mM NADH, 4.2 mM GSH, 0.4 mM ADP, 10 units lactate dehydrogenase and 2 units pyruvate kinase in 40 mM glycylglycine buffer, pH 7.4. Phosphoenolpyruvate carboxylase (EC 4.1.1.31) was assayed as described for the carboxylation reaction above except that ADP was omitted. Previously described methods were used for the assay of ribulosebisphosphate carboxylase (EC 4.1.1.39) [6], fructose-1,6-bisphosphatase (EC 3.1.3.11) [6], isocitrate lyase (EC 4.1.3.1) [3], and fumarate hydratase (EC 4.2.1.2) [3]. Contamination of the partially purified phosphoenolpyruvate carboxykinase was assessed by measuring enzyme activities by following $NADP^+$ reduction or NADH oxidation. All reaction mixtures were of 3.0 ml of 40 mM glycylglycine buffer, pH 7.4, that contained 4.2 mM GSH and 1 mM $MnCl_2$. The other components were: NADP-malic enzyme (EC 1.1.1.40), 5 mM malate and 2.4 mM $NADP^+$; pyruvate kinase (EC 2.7.1.40), 2 mM phosphoenolpyruvate, 0.4 mM ADP, 0.35 mM NADH and 10 units lactate dehydrogenase; malate dehydrogenase (EC 1.1.1.37), 0.5 mM oxaloacetate and 0.35 mM NADH; lactate dehydrogenase (EC 1.1.1.27), 1 mM pyruvate and 0.35 mM NADH. Protein was measured by the method of Warburg and Christian [10].

Metabolism of [^{14}C]acetate. Cotyledons were severed at their base from the

seedlings. A thin slice (5 × 3 mm; fresh weight, approx. 4 mg) was cut off the abaxial surface of each cotyledon and replicate samples, each of six cotyledons, were prepared. The samples given 3-mercaptopicolinic acid were pre-treated by incubation on filter paper (diameter 5 cm) in Petri dishes. The filter paper was moistened with 0.5 ml 2 mM 3-mercaptopicolinoc acid in 0.02 M KH_2PO_4, pH 5.2. After 30 min each sample was transferred to a 100 ml Erlenmeyer flask fitted with a centre well. In the flask the cotyledons were put on a filter paper (diameter 5 cm) to which had been added 0.5 ml 0.02 M KH_2PO_4, pH 5.2, that contained 3-mercaptopicolinic acid at 2 mM and 3.3 μCi [1-^{14}C]- or [2-^{14}C]acetate at 0.25 mM. The flasks were then incubated for 4 h. The control samples received exactly the same treatment except that the 3-mercaptopicolinic acid was omitted from both the pre-treatment and the 4 h incubation. The cotyledons were all placed cut surface down, and all incubations were in the dark at 25°C. The above procedure was adopted because we found that the oxygen uptake of marrow cotyledons is so high in relation to the diffusion coefficient of oxygen in water that suspension of the cotyledons in aqueous solutions limits respiration and interferes with gluconeogenesis. Measurements, by Warburg's direct manometric method, showed that our procedure did not alter the rates of either oxygen uptake or carbon dioxide production of the cotyledons.

$^{14}CO_2$ produced during the incubation was collected in alkali in the centre well. At the end of the incubation the cotyledons were lifted off the filter paper, blotted with fresh filter paper, and killed with boiling 80% (v/v) aq. ethanol. The ^{14}C that remained in the Erlenmeyer flask, and that removed by blotting, was counted and summed for each sample. The difference between the value obtained and the amount of ^{14}C supplied to each sample is taken as an estimate of uptake of [^{14}C]acetate. After being killed, each sample was extracted by boiling, successively, in 80% (v/v) aq. ethanol, 50% (v/v) aq. ethanol, water, and 80% (v/v) aq. ethanol. All the extracts were combined and evaporated under reduced pressure at 35°C. Glacial acetic acid (50 ml) was added to the residue and the resulting suspension was evaporated as above: this procedure was repeated thrice in order to remove any unmetabolized [^{14}C]-acetate. The water-soluble substances in the extract were then fractionated by ion-exchange and paper chromatography as described previously [5]. The solvent for the paper chromatography of the acidic components was butanol/formic acid/water (4 : 1 : 5, v/v), and that for the basic components was phenol/water (3 : 1, v/v), the water contained 4 mM EDTA and 4% (v/v) glacial acetic acid. For the separation of glycine from serine the above solvent was made 0.5% with respect to 20 M ammonia.

Results

Partial purification of phosphoenolpyruvate carboxykinase

Table I summarizes the results of a typical partial purification of phosphoenolpyruvate carboxykinase. Elution from the DEAE-Sephadex gave a number of fractions of high specific activity. Attempts to concentrate the activity in these fractions by adding $(NH_4)_2SO_4$ were unsuccessful. Thus, only one of these fractions was taken to the next stage of purification. In each experiment, the

TABLE I

PARTIAL PURIFICATION OF PHOSPHOENOLPYRUVATE CARBOXYKINASE FROM MARROW COTYLEDONS

About 30 g of cotyledons were extracted. The carboxylation reaction was assayed: 1 unit represents the transformation of 1 μmol substrate/min.

Step	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Yield (%)	Purification (-fold)
Crude extract	150	2950	173	0.06	100	0
0—50% $(NH_4)_2SO_4$	20	672	168	0.25	97	4
Calcium phosphate gel followed by precipitation with $(NH_4)_2SO_4$	3	85	74	0.87	43	15
DEAE-cellulose chromatography fraction 16	6	2.7	12	4.44	7	74
Sephadex G-200	2.3	0.58	2.8	4.83	1.6	81

fraction chosen was the one that was found to have the lowest activity of malate dehydrogenase. Our final preparations of phosphoenolpyruvate carboxykinase had a specific activity that was at least 80 times that of the initial extract. We detected no activity of malate dehydrogenase, NADP-malic enzyme, lactate dehydrogenase, pyruvate kinase, or phosphoenolpyruvate carboxylase in these final preparations.

With the exception of GSH, the activity of the partially purified phosphoenolpyruvate carboxykinase, when assayed in either direction, was completely dependent upon each component of the assay mixtures. Omission of GSH reduced both the forward and back reactions by 20%. The concentrations of the components of the assay mixtures are those that gave the optimum rates. With the other reactants at their optimum concentrations, Michaelis constants were determined from Lineweaver-Burk plots and were: oxaloacetate, 56 μM; ATP, 119 μM; $MnCl_2$ 0.45 mM. The rate of the decarboxylation reaction showed a normal hyperbolic dependence on the concentrations of oxaloacetate and ATP. Decarboxylation was optimum at pH 7.4, and the activities at pH 7.0 and pH 7.8 were 80% of the maximum rate. Carboxylation was optimum at pH 6.7 and the activities at pH 6.0 and pH 7.0 were 75% of the maximum rate. When decarboxylation and carboxylation were measured at their pH optima in the same extract, the rate of the former was 77% of that of the latter. Substitution of 1 mM Mg^{2+} for Mn^{2+} gave no detectable activity in either direction. Replacement of ADP with either 2 mM UDP or 2 mM GDP also resulted in no activity. The rate of the decarboxylation reaction was not significantly affected by: 2 mM ADP, 2 mM AMP, 0.5 mM $NADP^+$, 1.0 mM NADH, 1 mM NAD^+, 1 mM succinate, 1 mM malate, 2 mM oxaloacetate, 2 mM citrate, 50 mM bicarbonate. The rate of the carboxylation reaction was not affected by: 2 mM succinate, 2 mM malate, 5 mM fructose 1,6-bisphosphate.

In order to determine the intracellular location of phosphoenolpyruvate carboxykinase we employed the technique of gentle extraction that we had used, successfully, to isolate proplastids from cotyledons of the same age [6]. The results (Table II) show that hardly any of the activity was sedimented at

TABLE II

DISTRIBUTION OF PHOSPHOENOLPYRUVATE CARBOXYKINASE IN HOMOGENATES OF MARROW COTYLEDONS

Cotyledons (10 g) were extracted with 0.167 M glycylglycine buffer (pH 7.4) that contained 0.4 M sucrose and 0.5 mM dithiothreitol. The homogenate was filtered through muslin and the filtrate was centrifuged at 2500 × *g*; the sediment was collected and the supernatant was centrifuged at 10 800 × *g* to give a second sediment and a final supernatant. Enzyme activity in the two sediments and the final supernatant was determined and is summed to give the total activity recovered. The activity of each enzyme in each fraction is given as nmol substrate consumed/min per fraction, and as a percentage of the total activity recovered. Each value is the mean ± S.E. of data from three extracts.

Enzyme	Activity recovered per fraction					
	Sediment at 2500 × *g*		Sediment at 10 800 × *g*		Supernatant at 10 800 × *g*	
	nmol/fraction	Percent of total	nmol/fraction	Percent of total	nmol/fraction	Percent of total
Phosphoenolpyruvate carboxykinase	217 ± 178	1.5 ± 1.1	121 ± 24	1.2 ± 0.3	11 280 ± 1706	97.3 ± 0.8
Ribulosebisphosphate carboxylase	409 ± 69	24.9 ± 2.2	51 ± 16	3.3 ± 1.1	1 163 ± 67	71.9 ± 1.5
Isocitrate lyase	2312 ± 243	36.8 ± 2.2	158 ± 15	2.6 ± 0.5	3 844 ± 503	60.6 ± 2.5
Fumarate hydratase	1249 ± 150	56.9 ± 2.7	96 ± 36	4.5 ± 1.6	843 ± 74	38.6 ± 1.1

either 2500 $\times g$ or 10 800 $\times g$. We studied the extent to which our fractionation technique yielded intact organelles. Ribulosebisphosphate carboxylase, isocitrate lyase, and fumarate hydratase were used as markers for intact proplastids, glyoxysomes, and mitochondria, respectively. The sediments contained appreciable proportions of these enzymes, and the distribution of the latter differed sharply from that of phosphoenolpyruvate carboxykinase (Table II).

Activity of phosphoenolpyruvate carboxykinase during germination

We measured the activity of the enzyme in cotyledons at different stages of germination. We did this to see if the activity was high enough to support gluconeogenesis and to see if it coincided with gluconeogenesis. We optimized the pH, and the concentration of each component of the assay mixture for extracts of cotyledons from both 2-day-old and 5-day-old seedlings. Our estimates (Table III) show low activity for the first 2 days of germination, then a dramatic increase between days 2 and 4 to a broad peak of activity that was maintained until about day 7, from which point it declined so that no activity was found by day 18. We could detect no activity in extracts of young leaves of marrow. We investigated whether the above differences in activity were due to the formation or liberation of inhibitors or activators during the preparation of the extracts. We did thus by comparing the activities found in extracts of cotyledons from 2-day-old seedlings, from 8-day-old seedlings, and from a mixture of equal weights of cotyledons of the two ages. We did a similar experiment with cotyledons from ungerminated seeds and 5-day-old seedlings. In both instances the activity recovered in the extracts of the mixtures did not differ significantly from the value predicted from measurements made on the separate components of the mixtures. Discrepancy between the observed and predicted values for the mixtures would indicate loss or activation of the enzyme during the preparation of the extracts.

TABLE III

ACTIVITY OF PHOSPHOENOLPYRUVATE CARBOXYKINASE IN COTYLEDONS OF MARROW SEEDLINGS

Samples (2 g) of cotyledons were homogenized in 40 mM glycylglycine buffer (pH 6.7) which contained 10 mM EDTA and 10 mM dithiothreitol. The homogenate was centrifuged at 100 000 $\times g$ for 30 min and the supernatant assayed for the carboxylation reaction. Values are means ± S.E. of measurements made on six separately grown samples of cotyledons. Fisher's P values are given for comparison of successive ages of seedlings. Values of 0.05 or less are considered significant. Values greater than 0.05 are given as not significant (n.s.).

Days germinated	Activity (nmol/min per cotyledon)	Fisher's P values
0	17 ± 2	0 vs. 2 < 0.02
2	39 ± 4	2 vs. 4 < 0.001
4	362 ± 34	4 vs. 5 < 0.01
5	768 ± 31	5 vs. 7 n.s.
7	711 ± 43	7 vs. 8 < 0.05
8	441 ± 39	8 vs. 11 < 0.05
11	208 ± 34	
18	none detected	

Effects of 3-mercaptopicolinic acid

In animals, 3-mercaptopicolinic acid has been shown to inhibit phosphoenolpyruvate carboxykinase in vitro [11] and gluconeogenesis in vivo [12]. If the marrow enzyme were similarly sensitive then 3-mercaptopicolinic acid would be a useful tool for investigating the role of the enzyme in the cotyledons. We determined the effects of this compound on the activity of the enzyme in unfractionated extracts of cotyledons from 5-day-old seedlings (Fig. 1). Substantial inhibition was observed at quite low concentrations.

In view of the above results, we determined the effects of the inhibitor on gluconeogenesis in vivo. The manner in which excised cotyledons metabolize [1-^{14}C]- and [2-^{14}C]acetate was used to reveal any such effects. Samples of cotyledons were incubated in [^{14}C]acetate either in the presence or absence of the inhibitor, and the detailed distribution of ^{14}C was determined after 4 h. Incubation of the samples as described in Materials and Methods led to appreciable and reproducible metabolism of [^{14}C]acetate and did not cause anaerobiosis. Estimates of the percentage of the supplied ^{14}C that was adsorbed by the samples gave values of 39.8 ± 1.2 for the controls and 46.9 ± 1.5 for the treated samples. Each of these values is the mean ± S.E. of estimates from four samples, and the difference is significant ($P < 0.02$). Although 3-mercaptopicolinic acid had this small effect on uptake of [^{14}C]acetate, it made no significant ($P > 0.05$) difference to the percentage of the adsorbed [^{14}C]acetate that was metabolized. Thus the amounts of ^{14}C recovered per fraction are expressed as percentages of the total ^{14}C metabolized by the sample. Analysis of the ^{14}C in the media at the end of the incubations showed that very little of it was present as metabolites that had leaked out of the cotyledons. For example, in the experiment reported in Table IV, the ^{14}C recovered in the medium in compounds other than [^{14}C]acetate was 7.0 ± 0.8% (mean ± S.E. for four samples) of the ^{14}C metabolized.

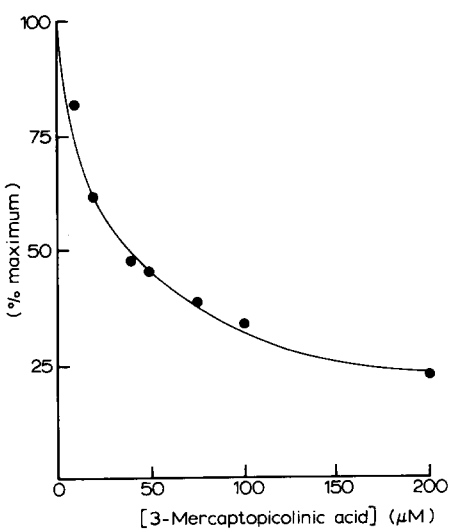

Fig. 1. Effect of 3-mercaptopicolinic acid on the activity of phosphoenolpyruvate carboxykinase in vitro. Data are from unfractionated homogenates assayed in the direction of carboxylation.

TABLE IV

EFFECTS OF 3-MERCAPTOPICOLINIC ACID ON METABOLISM OF [1-^{14}C]- AND [2-^{14}C]ACETATE BY MARROW COTYLEDONS

Samples of six cotyledons were incubated as described in Materials and Methods. After 4 h in [^{14}C]acetate the samples were killed with boiling 80% ethanol, and extracted successively with 80% ethanol, 50% ethanol and water. Unmetabolized [^{14}C]acetate was removed from the combined extracts which were then fractionated by ion-exchange and paper chromatography. The ^{14}C recovered as $^{14}CO_2$, in fats, in the water-soluble substances, and in the water-insoluble substances was summed to give the amount of ^{14}C metabolized by the samples.

Fraction	Percentage of metabolized ^{14}C recovered per cell fraction			
	[1-^{14}C]Acetate		[2-^{14}C]Acetate	
	Control	3-Mercapto-picolinic acid	Control	3-Mercapto-picolinic acid
CO_2	30.1	31.5	3.2	9.6
Fats	15.2	9.6	13.5	13.6
Water-insoluble substances	18.0	20.3	30.8	18.7
Water-soluble substances	27.4	32.2	46.2	52.3
Neutral components	10.0	3.5	21.8	7.1
Sucrose			9.6	0.4
Stachyose			5.6	2.2
Glucose			1.1	0.5
Fructose			0.7	0.4
Basic components	7.1	9.3	9.0	19.7
Alanine			2.6	7.0
Glutamate			1.6	5.1
Valine + histidine			1.5	2.1
Aspartate			0.8	2.8
Serine			0.8	0.5
Glycine			0.5	0.3
Acidic components	8.2	13.6	10.5	19.3
Malate			5.4	12.1
Succinate			2.2	3.8
Fumarate			0.7	1.1
Citrate + isocitrate			0.3	0.5
10^{-6} X Total ^{14}C absorbed (dpm)	3.11	3.13	3.08	3.74
10^{-6} X Total ^{14}C metabolized (dpm)	1.47	2.18	1.82	2.01

The effects of 3-mercaptopicolinic acid on the metabolism of [^{14}C]acetate are shown in Table IV. These labelling patterns were obtained consistently except that in no other experiment did we detect any effect of the inhibitor on the labelling of the lipids. Examination of the amounts of ^{14}C recovered in the different fractions shows that the labelling patterns were not seriously affected by losses during the analyses. We stress the following aspects of these results. In the control samples a substantial proportion of the metabolized [^{14}C]acetate was converted to sugars. There was also appreciable labelling of amino acids and organic acids. The major effects of 3-mercaptopicolinic acid on this metabolism were as follows. First, $^{14}CO_2$ production from [2-^{14}C]acetate was trebled but that from [1-^{14}C]acetate was not altered. Second, there was a reduction in the contribution of C-2, but not in that of C-1, to the insoluble material. Third, although the percentage of the ^{14}C recovered as soluble substances was not changed much, a slight increase for both carbons, the detailed

distribution of ^{14}C within this fraction was altered radically. The percentages of C-1, and of C-2, that were recovered as neutral compounds were reduced by two thirds. Detailed analysis for C-2 showed that this reduction occurred almost entirely at the expense of sugars. In contrast there was an increase in the contributions of both carbons to the acidic and basic components. For the basic substances this increase can be accounted for almost completely as alanine, glutamate and aspartate. Most of the increase in the acidic fraction occurred as malate and, to a lesser extent, succinate.

We investigated whether 3-mercaptopicolinic acid inhibited a number of enzymes, other than phosphoenolpyruvate carboxykinase, that might affect the labelling pattern from [^{14}C]acetate. The experiments were carried out with unfractionated extracts of cotyledons from 5-day-old seedlings. We found that 0.1 mM 3-mercaptopicolinic acid had no detectable effect on the activities of isocitrate lyase, fructose-1,6-bisphosphatase, pyruvate kinase, and ribulosebisphosphate carboxylase.

Discussion

Our preparations of phosphoenolpyruvate carboxykinase were free of enzymes capable of interfering with the assay. The complete dependence of the activity of our preparations upon each substrate, and upon each coupling enzyme, demonstrates that this activity was that of phosphoenolpyruvate carboxykinase. The enzyme from marrows closely resembles those described from leaves of C_4 plants [13,14], and all three plant enzymes described so far are similar to the enzyme present in gluconeogenic tissues of animals. The only major difference noted is that ATP is the preferred substrate for the plant enzymes whereas the mammalian enzyme can use GTP and ITP but not ATP [9]. The general similarity of all the enzymes is demonstrated by the absolute requirement for Mn^{2+}, the low K_m for oxaloacetate and for ATP, the pH optimum, and the lack of any significant regulatory response to metabolites.

The low K_m of the marrow enzyme for oxaloacetate and for ATP, and the high concentration of bicarbonate needed for the carboxylation reaction, are consistent with the role proposed for this enzyme in gluconeogenesis in fatty seedlings. This proposal is supported by our measurements of the activity of the enzyme during germination (Table III). We suggest that these measurements reflect the maximum catalytic activities of the cotyledons. First, the assays were carefully optimized. Second, the values obtained for the mixed samples were so close to the predicted values that it is unlikely that our estimates were affected by loss or activation of the enzyme in the homogenates. In marrow cotyledons under the conditions of our experiments, gluconeogenesis does not begin until after 2 days germination, then it increases rapidly to reach a peak between 5 and 8 days from planting [5]. Thus, the variation in the activity of phosphoenolpyruvate carboxykinase during germination correlates extremely closely with the extent of gluconeogenesis. The maximum estimate of the rate of gluconeogenesis is 60 nmol hexose formed/min per cotyledon [5]. The maximum activity of the enzyme is ten times this value (Table III). Consequently there can be little doubt that there is sufficient enzyme present to support all of the gluconeogenesis in the cotyledon.

Proof of the role of phosphoenolpyruvate carboxykinase in gluconeogenesis in marrows is provided by our experiments with 3-mercaptopicolinic acid. The manner in which excised cotyledons of marrows metabolize [^{14}C]acetate has been reported [5]. Thus the labelling of the control samples requires little comment. The present, in contrast to the previous, results show heavier labelling of the sugars at the expense of the amino acids and organic acids. We attribute this difference to the fact that in the earlier work we supplied the [^{14}C]acetate to cotyledons suspended in an aqueous medium [5]. This procedure is likely to have reduced gluconeogenesis through reduction of the oxygen tension in the cotyledons. The effects of 3-mercaptopicolinic acid on the metabolism of [^{14}C]acetate, specifically the marked reduction in the labelling of the sugars, show that this compound caused substantial inhibition of gluconeogenesis. The observation that the labelling of sucrose was restricted more severely than that of stachyose and hexose probably reflects gradual penetration of the inhibitor and the fact that sucrose is the more immediate product of gluconeogenesis as the other sugars are formed from it. The decreased contribution of C-2 to the insoluble fraction probably reflects reduced conversion of acetate to polysaccharide, and is further evidence of inhibition of gluconeogenesis. The distortion of the labelling patterns indicates that the inhibition of gluconeogenesis was due to restriction of the conversion of oxaloacetate to phosphoenolpyruvate. Such restriction would divert oxaloacetate, normally destined for conversion to phosphoenolpyruvate, to malate, aspartate, the tricarboxylic acid cycle, and related reactions. In marrow cotyledons the tricarboxylic acid cycle is active during gluconeogenesis in support of the biosyntheses necessary for the formation of photosynthetic tissue [5]. The detailed distribution of label from [2-^{14}C]acatate agrees with the above predictions. Treatment with the inhibitor reduced the percentage of the metabolized ^{14}C that was recovered in the neutral plus insoluble fractions from 53 to 26. All but a very small fraction of this reduction can be accounted for by the increased labelling of alanine, glutamate, aspartate, malate, succinate and CO_2. The greater labelling of malate and aspartate would result directly from increased availability of oxaloacetate, the rise in the labelling of glutamate and CO_2 from metabolism of oxaloacetate via the tricarboxylic acid cycle, and the increase in alanine is probably the result of malic enzyme activity. We suggest that 3-mercaptopicolinic acid inhibits gluconeogenesis in marrow cotyledons by reducing the activity of phosphoenolpyruvate carboxykinase.

From the evidence in this paper, and the results of the feeding experiments carried out by Canvin and Beevers [15], we conclude that the conversion of oxaloacetate to phosphoenolpyruvate by phosphoenolpyruvate carboxykinase is an essential step in gluconeogenesis in fatty seedlings. The intra-cellular location of this step is indicated by our cell fractionation experiments (Table II). Significant proportions of the ribulosebisphosphate carboxylase, isocitrate lyase, and fumarate hydratase of the extracts were recovered in the particulate fractions. Detailed study of these fractions has shown that they contain intact proplastids, glyoxysomes, and mitochondria [6]. As these fractions were almost devoid of phosphoenolpyruvate carboxykinase activity, it is unlikely that any appreciable proportion of this enzyme is located in any of the above organelles in marrow cotyledons. Thus, we suggest that the conversion of oxaloacetate to

phosphoenolpyruvate occurs in the soluble phase of the cytoplasm in higher plants.

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