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CO_2 Metabolism in Corn Roots. I. Kinetics of Carboxylation and Decarboxylation[1]

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Summary. The kinetics of $^{14}CO_2$ carboxylation and decarboxylation in corn root tips were determined to ascertain the sequence of product formation and subsequent utilization, and to obtain further evidence to predict the enzymes mediating the carboxylation and decarboxylations. The carboxylation data indicated that the first product was oxaloacetate followed by malate and aspartate. Malate was the first stable product which could be detected. Decarboxylation data indicated that a large fraction of the $^{14}CO_2$ release and turnover of ^{14}C was accountable for by a decrease in malate; however, essentially all labeled amino acids turned over rapidly and at a greater rate than organic acids. The data generally support the hypothesis that CO_2 fixation in corn root tips is via P-enolpyruvate carboxylase and malic dehydrogenase and that subsequent malate metabolism is for the most part by direct decarboxylation, possibly by the malic enzyme.

In previous work concerning CO_2 metabolism in root tips, we observed a rapid turnover of the fixed $^{14}CO_2$ which was correlated with a decrease in the main product, malate (17, 18). A 3 enzymic sequence involving P-enolpyruvate (PEP) carboxylase, malic dehydrogenase, and the malic enzyme was postulated to account for these observations. Subsequently, data were obtained to support the hypothesis (17). This paper reports in vivo kinetic data and in vitro $^{14}CO_2$ tracer data which further corroborate the hypothesis.

Materials and Methods

Plant Materials. Corn (Burpee's Golden Bantam Cross) root tips (2 cm) were obtained from 2 and one-half day old seedlings grown at 25° between moist paper toweling. The seeds were soaked in aerated, distilled water for 6 hours before planting in covered trays.

In Vitro Studies. Soluble fraction enzyme homogenates were prepared by grinding 10 g of root tips with acid washed sand in 2 volumes of 10 mM tris- 0.5 M sucrose buffer (pH 7.4), centrifuging the homogenate at 10,000 × *g* for 30 minutes to remove particulate fractions, and dialyzing against 2 changes of 10 mM tris (pH 7.4) overnight. A second centrifugation results in a several-fold purification of enzymes as estimated from the loss of protein. All operations were completed at 2 to 4°.

Enzymatic assays were conducted by radioisotope analysis. PEP carboxylase was assayed by adding 10 μmoles of P-enolpyruvate (trisodium salt), 10 μmoles $MgCl_2$, 1 μmole of $NaH^{14}CO_3$ (48 μc/μmole) to 9 ml (about 3 g fr wt of tissue) of enzyme homogenate (pH 7.4). The total volume was 12 ml. The reaction mixture was incubated for 15 minutes at 25° in a water-bath shaker. The reaction was stopped by adding an equal volume of 2,4-dinitrophenylhydrazine (25 mg hydrazine/ml 6 N HCl) to form the hydrazone derivative of the product, oxaloacetate. The hydrazones were extracted according to Aronoff (1) and aliquots were taken for counting of radioactivity with a liquid scintillation counter (16). Additional samples were chromatographed on silica gel H (16) in a petroleum ether: ethyl acetate: acetic acid (13:7:2) developer [modified after Ronkainen (13)]. The oxaloacetate derivative was located on the chromatoplates by co-chromatography with known oxaloacetate-hydrazones as well as with α-ketoglutarate and pyruvate derivatives. Radioactivity was located on the chromatoplates by autoradiography, and ^{14}C associated with oxaloacetate was determined by scraping the gel directly into liquid scintillation vials. These procedures were previously described (16). Using the specific activity of added $NaH^{14}CO_3$, the data were converted to μmoles ^{14}C fixed per hour per g fresh weight tissue.

The coupled reaction involving PEP carboxylase and malic dehydrogenase to form malate was assayed similarly to PEP carboxylase (see above) except that 10 μmoles of NADH were added to the reaction mixture. This reaction was stopped with 2 ml of 88% formic acid, freeze-dried to remove

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the formic acid, and the product, malate, was prepared by ion exchange and thin-layer chromatography as previously described (16). Malate was located on the thin-layer plates by co-chromatography with known malate and radioactivity was located by autoradiography (16). Assay of the radioactivity associated with malate, the only product, was conducted as described above for oxaloacetate. The data were converted to μmoles ^{14}C fixed into malate per hour per g fresh weight.

The malic enzyme was assayed similarly to PEP carboxylase (see above) except that the reaction mixture contained 2 μmoles NADP, 10 μmoles $MgCl_2$, and 1.78 μmoles DL-malate-^{14}C (2.2 μc/μmoles). The reaction was stopped with 2,4-dinitrophenylhydrazine, and the product, pyruvate, was prepared and chromatographed in the same manner as oxaloacetate. The data were converted to μmoles pyruvate synthesized per hour per g fresh weight (based on specific activity of malate-^{14}C added).

Satisfactory assays for these reactions can be conducted in a total volume of 3 ml with 0.1 ml of enzyme preparation and 2 μmoles PEP, 10 μmoles $MgCl_2$, 0.25 μmoles NADH or NADP, and 1 μmole of $NaH^{14}CO_3$ or DL-malate-^{14}C (specific activities as given above). The reactions are run for 5 minutes at 25° in 50 mM tris (pH 7.4).

In Vivo Studies. $^{14}CO_2$ carboxylation kinetics were determined by incubating 0.2 g of 2 cm root tips in 50 μc of $NaH^{14}CO_3$ (52 μc/μmole) in 2 ml of 10 mM tris buffer (pH 7.4) for periods of 1 minute to 2 hours.

Keto acids were determined by killing the tissue in 2 ml of 2,4-dinitrophenylhydrazine (25 mg/ml 6 N HCl), grinding in a teflon-glass tissue grinder, and extracting and chromatographing as described under in vitro studies. The products of the carboxylation were located on the silica gel plates by co-chromatography with known oxaloacetate-, α-ketoglutarate-, and pyruvate-hydrazone derivatives. Radioactivity was located by autoradioactivity, and ^{14}C activity associated with each product was determined by scraping the product with silica gel into liquid scintillation vials (see in vitro studies above and ref. 16).

Non-keto organic acids were determined by quickly washing the tissue with water after the incubation period, freezing in liquid N_2, grinding to a fine powder while frozen, extracting with chloroform:methanol:water (16), separating the methanol:water fraction into anion, cation, and neutral fractions by ion exchange chromatography (3), and chromatographing the anion fraction on silica gel as previously described (16). Organic acids were determined by R_F and co-chromatography with known acids. Malate, after elution from the chromatoplates with 50 mM tris buffer (pH 7.4), was further verified by decarboxylation with a preparation from *Lactobacillus arabinosus* according to Korkes (6). Succinate, after elution from the chromatoplates, was assayed with succinic oxidase (21). Aconitate was determined as the trans derivative by R_F, co-chromatography with known aconitate, and by its distinctive brown color and fluorescence after spraying the chromatoplate with acetic anhydride-pyridine reagent. *Cis*-aconitate is quantitatively converted to trans by the extraction and ion exchange chromatography procedure, thus it was not possible to differentiate between the 2 isomers.

Amino acids were prepared similarly to the organic acids (from the cation fraction) except chromatography was in several different developing systems. One dimensional thin-layer chromatography of amino acids was conducted on silica gel H in a chloroform:methanol:NH_4OH (50:50:22–22 ml of 15 ml 30 % NH_4OH plus 10 ml H_2O) similar to the method of Honegger (5) and by a 2-dimensional method involving electrophoresis in 1 direction and chromatography in the other. The 2-dimensional method was similar to that described by Honegger (5) except that the absorbent was a mixed layer of cellulose and silica gel H according to Turner and Redgwell (19). Amino acids were verified by co-chromatography and their distinctive color reactions with ninhydrin. Radioactivity in each amino acid was determined as described for the organic acids.

Decarboxylation kinetics were determined by incubating 0.2 g of root tips in 2 ml of 10 mM tris buffer (pH 7.4) plus 50 μc of $NaH^{14}CO_3$ (52 μc/μmole) for 2 hours and then quickly washing with distilled water and transferring to a non-$H^{14}CO_3^-$ buffer for periods up to 2 hours. The preparation and determination of products was identical to that described above.

The total ^{14}C incorporated (water soluble, lipid, RNA, DNA, and insoluble) into components of the root tip was determined by Tsay et al. (20) modification of Schmidt and Thannhauser's (14) method. Root tips (0.35 g, 2 cm long) were incubated in 5 ml of 50 mM tris (pH 7.4) with 50 μc of $NaH^{14}CO_3$ (52 μc/μmole) for periods up to 4.3 hours. The reaction was stopped by freezing in liquid N_2 and ground to a fine powder before extraction (20). The ^{14}C activity incorporated into each fraction was determined by liquid scintillation counting of an aliquot from each sample.

Results

Determination of Reaction Products. In order to verify the synthesis of oxaloacetate, malate, and pyruvate, enzyme homogenates were incubated with ^{14}C labeled substrates. When the incubation was conducted with PEP, Mg^{2+}, and $H^{14}CO_3^-$, oxaloacetate was the only product labeled. If the reaction was coupled with malic dehydrogenase by adding NADH, malate was synthesized. Verification of the malic enzyme and the product, pyruvate,

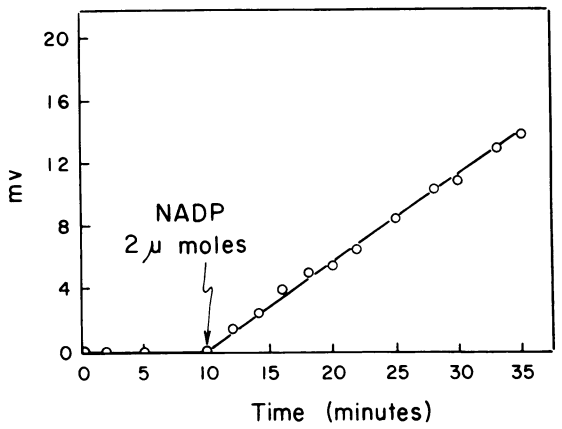

FIG. 1. Decarboxylation of purified malic acid by the malic enzyme. Malic acid was synthesized by an enzyme homogenate prepared from corn root tips. The synthesis-incubation mixture contained 10 μmoles PEP, 10 μmoles $MgCl_2$, 10 μmoles NADH, 1 μmole $NAH^{14}CO_3$ (48 μc/μmole), and 9 ml of enzyme extract made up to a total volume of 12 ml with 50 mM tris buffer, pH 7.4. The reaction was run for 15 minutes at 25°. The malic acid synthesized was purified by thin-layer chromatography as described in the text, and an aliquot (0.5 μc) was added to 9 ml of enzyme prep containing 10 μmoles $MgCl_2$ (total volume = 12 ml). Two μmoles NADP were added to start the reaction. 1295 dpm/mv.

was made with NADP, Mg^{2+}, and malate-3-^{14}C; the labeled product was pyruvate. The rates of reactions considering only added labeled substrates were 1.36 μmoles g^{-1} fresh weight $hour^{-1}$ (PEP carboxylase), 0.52 μmole g^{-1} fresh weight $hour^{-1}$ (coupled PEP carboxylase and malic dehydrogenase), and 0.19 μmole g^{-1} fresh weight $hour^{-1}$ (malic enzyme).

Presumably, the malate formed by coupling PEP carboxylase and malic dehydrogenase should be mostly L-malate-4-^{14}C (2). Thus, the products of the decarboxylation of the malate should be unlabeled pyruvate and $^{14}CO_2$. The malate synthesized from the coupled reaction was purified by silica gel thin-layer chromatography, eluted from the gel with 10 mM tris buffer (pH 7.4), and incubated with the soluble enzyme homogenate. The reaction was followed in a closed system with a Cary Model 31 electrometer and ion chamber as previously described (18). When only Mg^{2+} and the enzyme homogenate were included, there was no $^{14}CO_2$ produced. Upon addition of NADP, $^{14}CO_2$ was generated linearly (fig 1). As noted above, the product of this reaction, when incubated in commercially prepared malate-3-^{14}C, was pyruvate-^{14}C. Thus these data indicate a positive assay for the malic enzyme and that the product of the coupled reaction, PEP carboxylase and malic dehydrogenase, will act as a substrate for the malic enzyme.

CO_2 Fixation Kinetics. The kinetics of keto acid formation after incubation in $H^{14}CO_3^-$ suggested that oxaloacetate was the first product of the in vivo carboxylation (fig 2). Other detectable labeled products were α-ketoglutarate and pyruvate. When the data were plotted as percent of total activity against time, oxaloacetate had a negative slope suggesting that it was the first product. The slower appearance of α-ketoglutarate and pyruvate indicated that they were secondary products of the carboxylation.

Under the conditions of our experiments, the kinetics of formation of stable water soluble components (organic and amino acids) were linear with time (fig 3). When the organic acid components were plotted as percent of total activity in the fraction against time, malic acid had a negative slope indicating that it was the first stable product of the carboxylation (fig 4). The other 2 easily detectable products, *trans*-aconitic and citric acids, increased initially and were, therefore, apparently secondary products. Both *trans*-aconitic and succinic acids have similar R_F values. The distinctive radioactive spot gives a slight positive succinic oxidase assay and less than 5% is converted to fumaric acid. The spot, however, gives a strong positive assay for *trans*-aconitic thus we have assumed that most of this compound is *trans*-aconitic acid. *Trans*-aconitic acid has previously been detected as a product of $^{14}CO_2$ fixation by corn root tips (10). After extended periods of incubation, labeled fumaric and isocitric acids could

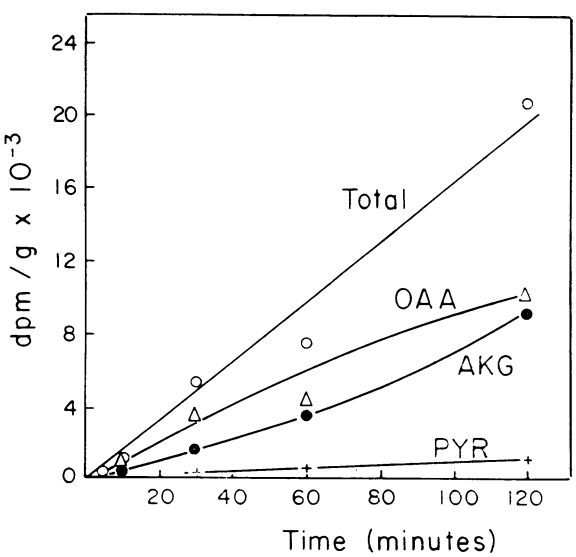

FIG. 2. In vivo carboxylation kinetics. Oxaloacetate (OAA) is apparently the first product. About 0.2 g of 2 cm long corn root tips were incubated in 50 μc (52 μc/μmole) of $NaH^{14}CO_3$ in 2 ml of 10 mM tris buffer (pH 7.4) for the times indicated. The 2,4-dinitrophenylhydrazones of the keto acids were chromatographed by thin-layer chromatography and the radioactivity associated with each product was determined by liquid scintillation counting. AKG = α-ketoglutarate, PYR = pyruvate.

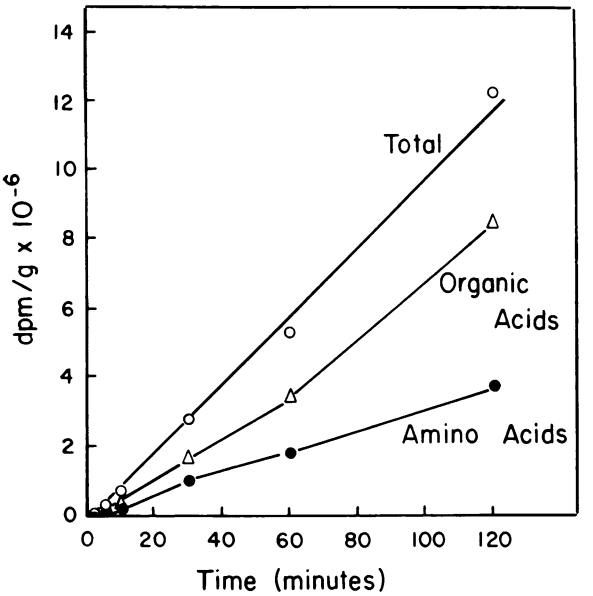

FIG. 3. In vivo carboxylation kinetics of stable water soluble components. About 0.2 g of 2 cm long corn root tips were incubated in 50 μc (52 μc/μmole) of $NaH^{14}CO_3$ for the times indicated. The products were extracted after freezing and grinding in liquid N_2, and separated by ion exchange chromatography. Samples from anion (organic acids), cation (amino acids), and neutral (carbohydrates) fractions were counted by liquid scintillation. After 1 minute only organic acids were detected. No activity was detected in the neutral fraction.

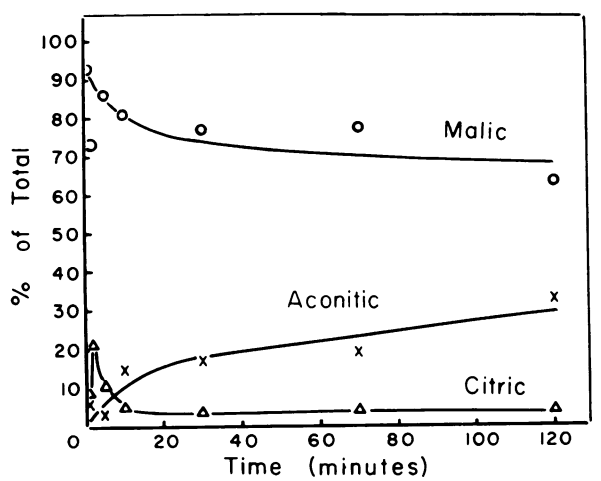

FIG. 4. In vivo carboxylation kinetics of stable organic acids. The organic acid fraction shown in figure 3 was chromatographed on silica gel and radioactivity associated with each product was determined. The negative slope of malic acid suggests it was the first organic acid product. The curve labeled aconitic acid contains some succinic acid.

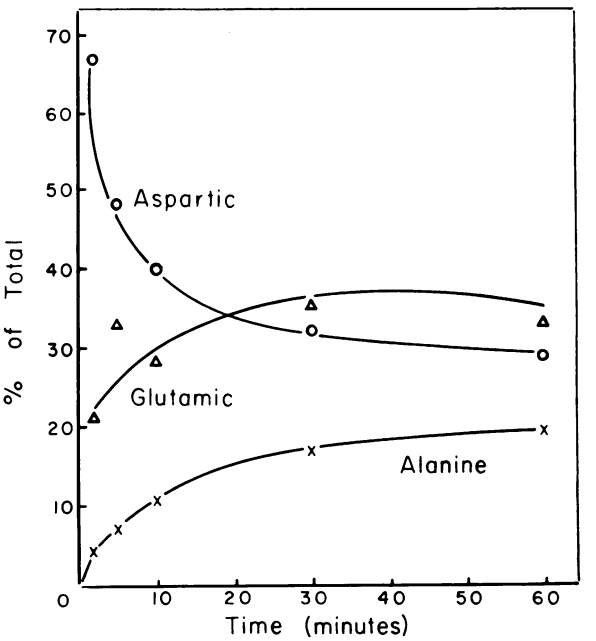

FIG. 5. In vivo carboxylation kinetics of amino acids. The amino acid fraction shown in figure 3 was chromatographed on silica gel. Radioactivity was determined by liquid scintillation counting. The negative slope of aspartic acid suggests it was the first product.

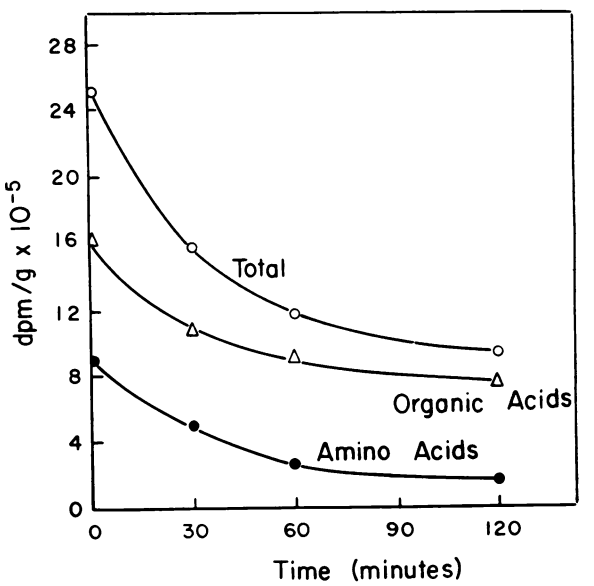

FIG. 6. In vivo decarboxylation kinetics. Both organic and amino acids are in active turnover pools. The analysis was conducted by incubating 0.2 g of 2 cm long root tips in 50 μc (52 μc/μmole) of $NaH^{14}CO_3$ in 2 ml of 10 mM tris buffer (pH 7.4) for 2 hours and then transferring to a non-$H^{14}CO_3$ buffer for the times indicated. Radioactivity was determined as described in figure 3 and the text.

be detected on the thin-layer plates, however the low activities made kinetic data difficult to interpret. Certainly, the latter were secondary products. Similarly, when the products in the amino acid fraction were plotted as a percent of total activity in the fraction against time, aspartic acid had a negative slope followed by glutamic acid and alanine with positive slopes (fig 5). Thus one must conclude that aspartic acid was the first stable amino acid formed during carboxylation. For extended periods of incubation, asparagine and glutamine could also be detected. One minute incubations resulted essentially in malic acid synthesis with no other detectable organic or amino acids.

Decarboxylation kinetics indicated that both amino acids and organic acids decayed at a rapid and logarithmic rate (fig 6). When plotted as a percent of remaining activity, the apparent rate of amino acid turnover was greater than that for organic acids (fig 7). The same observation was

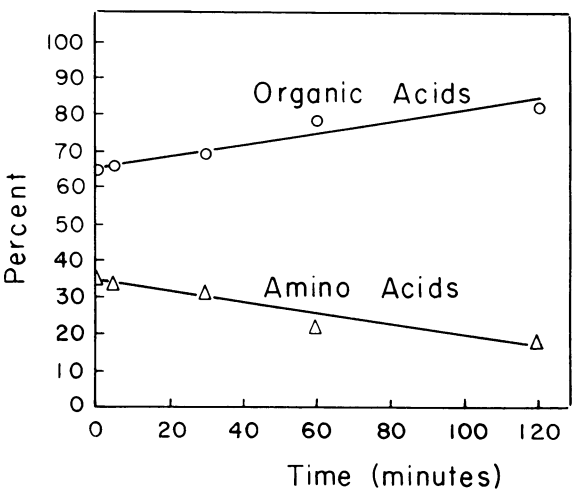

FIG. 7. In vivo decarboxylation kinetics of organic and amino acids, plotted as percent remaining against time. Amino acids apparently turn over at a rate greater than organic acids. The data are from figure 6.

made with *Opuntia* roots (18). The turnover of organic acid products was reflected primarily in the decay of labeled malic acid (fig 8) whereas the *trans*-aconitic activity remained essentially constant. Since between 60 and 70 % of the total label was in the organic acid fraction after a 2 hour incubation (fig 2), the significant portion of the turnover of ^{14}C reflects the rate of decrease of labeled malic acid.

A long term incubation was conducted in an attempt to determine how much ^{14}C was eventually transferred to other stable components within the corn root tip. Even after 4.3 hours of incubation in $H^{14}CO_3^-$, relatively insignificant levels of radioactivity were detected in lipid, nucleotides, protein, or polysaccharides (table I).

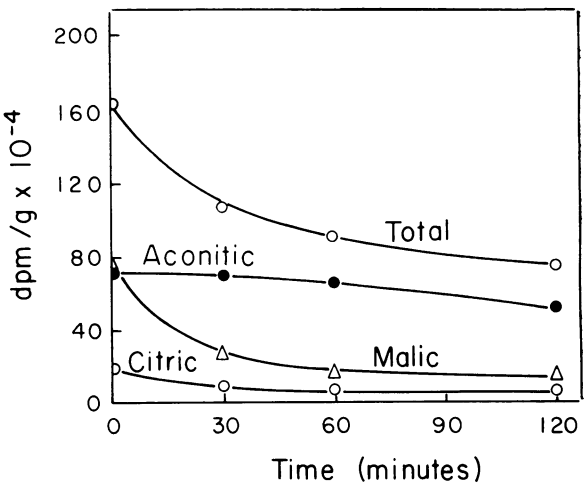

FIG. 8. In vivo decarboxylation kinetics of organic acid fraction. The loss in activity in the fraction reflects primarily the turnover of malic acid. The curve labeled aconitic contains some succinic acid. The reaction was conducted by incubating 0.2 g of root tips in 50 μc (52 μc/μmole) of $NaH^{14}CO_3^-$ for 2 hr, then transferring to a non-$H^{14}CO_3$ buffer for the indicated times. The radioactivity in each acid was determined by liquid scintillation counting after thin-layer chromatography.

Table I. *Distribution of ^{14}C in Corn Root Tips*

0.35 g Of 2 cm long root tips were incubated at 25° in 50 μc (52 μc/μmole) of $H^{14}CO_3^-$ in 5 ml of 50 mM tris buffer (pH 7.4) for 4.3 hr. The products were assayed by extraction with a modified Schmidt-Thannhauser method and aliquots of each fraction were counted with a liquid scintillation counter.

Fraction	dpm/g fr wt	%
Water soluble	4,536,000	99.50
Lipid	4130	0.09
RNA	16,400	0.36
DNA	860	0.02
Insoluble*	1360	0.03
Total	4,558,750	100.00

* Assumed to be protein and polysaccharides.

Discussion

Reaction Products. In a previous paper, we speculated that a consequence of CO_2 fixation in corn root tips was a transhydrogenation reaction converting NADH to NAD^+, and $NADP^+$ to NADPH. The proposed reaction proceeded by coupling PEP carboxylase, malic dehydrogenase, and the malic enzyme. In vitro spectrophotometric data were subsequently obtained to support the hypothesis (17). Walker (22), in a review paper concerning succulents, also suggested that the malic enzyme was a decarboxylating enzyme. Verification of specific reaction products, i.e., oxaloacetate, malate, and pyruvate was, however, not made. The in vitro incubations with enzyme homogenates

reported in this paper tend to support the hypothesis of the series reaction. Further substantiation was obtained by showing that the malate synthesized by PEP carboxylase and malic dehydrogenase could be decarboxylated by a malic enzyme reaction in the same homogenate. Thus, these data also support, but of course do not prove, the hypothesis of a series coupling of the 3 enzymic system.

The data indicated that the order of reactivity in the homogenates was PEP carboxylase > PEP carboxylase + malic dehydrogenase > malic enzyme. If, however, malic dehydrogenase was assayed directly using oxaloacetate as a substrate, it was far more active than PEP carboxylase (by 1–2 orders of magnitude). The coupled reaction, PEP carboxylase and malic dehydrogenase, would certainly be limited by the slower of the two, PEP carboxylase, and it is, therefore, not surprising to find the above order.

Carboxylation Kinetics. In vivo carboxylation kinetics support the hypothesis that PEP carboxylase could be a main CO_2 fixation enzyme in corn root tips. Oxaloacetate is apparently the first product followed by the stable products, malic and aspartic acids, in the organic and amino acid fractions respectively. Furthermore, the in vitro carboxylating activity of PEP carboxylase is sufficient to account for all of the in vivo $^{14}CO_2$ uptake (16.4×10^4 dpm g^{-1} fr wt min^{-1} for the homogenate against 9.0×10^4 dpm g^{-1} ft wt min^{-1} for the intact root tips).

It seems reasonable to assume that in vivo the oxaloacetate formed by direct carboxylation is converted rapidly to malic acid by malic dehydrogenase, and although not indicated directly by these experiments, oxaloacetate is probably also converted to aspartic acid via an amination or transamination reaction. Thus the carboxylation is evidently similar to most reported plant tissues (12). These data do not suggest mechanisms for the in vivo synthesis of the other prominent products, however, the evidence does suggest that they are secondary components. An attempt to outline metabolic pathways for their synthesis would be entirely speculative and, therefore, will not be attempted.

Decarboxylation Kinetics. The relatively rapid release of $^{14}CO_2$ from root tissue after $^{14}CO_2$ fixation as previously reported (17, 18) is reflected in the decarboxylation kinetics reported in this paper. Disregarding the amino acid decay data, the loss of label in the organic acid fraction seems largely due to the loss of labeled malate. The other prominent products remain relatively stable with respect to ^{14}C label and thus it is unlikely that much malate is converted directly to them during the post-incubation period. The data strongly suggest that malate is decarboxylated directly, and it is 1 thesis of this paper that the decarboxylation is largely via the malic enzyme. Other possibilities of course exist, however, a reversal of the presumed carboxylation pathway is extremely unlikely because of the kinetics of the reactions (4).

The data do not suggest a particular reaction sequence to account for the loss of label in amino acids. Two obvious possibilities are transamination resulting in ^{14}C labeled keto acids or direct decarboxylation. In any case, the data do indicate that the amino acids are in active turnover pools.

Marsh et al. and MacLennan et al. (11) reported 2 malate pools in plant tissues. Furthermore, Lips and Beevers (8, 9) have recently reported data obtained with corn root tips indicating that malic acid formed via CO_2 fixation and that formed via acetate assimilation are in separate metabolic pools with little interchange except under certain stress conditions such as when tricarboxylic acid cycle intermediates are limiting respiratory activity. Their methods of experimentation also resulted in data indicating a rapid turnover of malic acid formed from CO_2 fixation. The data and conclusions in this paper do not contradict theirs.

Equilibration of Initial Products with Other Components. An important observation was the lack of significant ^{14}C equilibration with products other than those initially formed. Essentially only a limited number of organic and amino acids were formed after carboxylation. Even after extended periods of incubation, relatively little activity was isolated in lipids, carbohydrates, polysaccharides, nucleotides, or proteins. The lack of labeled carbohydrates by dark CO_2 fixation has been reported by several workers (e.g., 7, 18), but the lack of significantly labeled protein is somewhat enigmatic. Splittstoesser (15) recently reported labeled aspartic and glutamic acids in the insoluble residue (protein) from $^{14}CO_2$ fixation by corn roots, but after 5 hours of incubation, the total activity in the insoluble residue was less than 1 % of the total fixed $^{14}CO_2$ (estimated from his table I). Thus our results are in general agreement with his.

In sum, the in vivo carboxylation kinetics followed with $^{14}CO_2$ suggested that oxaloacetate was the initial product with the subsequent products, malate and aspartate. The in vitro $^{14}CO_2$ carboxylating activity of PEP carboxylase was sufficient to account for all of the in vivo $^{14}CO_2$ uptake. Further, the data indicated that much of the turnover of $^{14}CO_2$ is reflected in the loss of labeled malate, and that the malic enzyme could mediate the decarboxylation reaction. The data are consistent with, but do not necessarily prove, the hypothesis that CO_2 metabolism in corn root tips is mediated, in part, by PEP carboxylase, malic dehydrogenase, and the malic enzyme.

Acknowledgments

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