CARBON DIOXIDE FIXATION INTO OXALACETATE IN HIGHER PLANTS[1,2]

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In 1938, Wood and Werkman (27) proposed that certain metabolic phenomena in bacteria could be explained in terms of the occurrence of a CO_2-fixing reaction. They suggested that CO_2 is added to pyruvate to form oxalacetate (OAA), as shown in equation (*1*). Since that time considerable information has been accumulated about the detailed mechanism

$$CO_2 + \text{pyruvate} \longrightarrow \text{OAA} \qquad (1)$$

of this "Wood-Werkman reaction." It was recognized early that simple addition of CO_2 to pyruvate occurred with such an unfavorable free energy change that some device for supplying chemical energy was essential if the reaction was to play a quantitatively important role in intermediary metabolism (7). Three different CO_2-fixing processes whereby this end can be achieved have been described to date. These are: a) the malic enzyme (or Ochoa) reaction, b) the phosphoenolpyruvate carboxykinase (or pepcarboxykinase) reaction, and c) the phosphoenolpyruvate carboxylase (or pepcarboxylase) reaction. All of these reactions involve the addition of CO_2 to a C-3 unit, to form a C-4 dicarboxylic acid; and all three reactions must be combined with some other reaction to give the net result shown in equation (*1*).

The focus of current interest has been partially on the detailed mechanism of these different enzyme reactions, and partially on their physiological interrelationships. Although the "malic" enzyme had been shown to be widely distributed (24), only limited information was available about the distribution of the other enzymes. The work described in the present paper was concerned mainly with the problems of assaying pepcarboxylase and pepcarboxykinase, and determining their reaction characteristics and distribution in higher plants. The reaction catalyzed by pepcarboxykinase, first described by Bandurski and Greiner (3, 4), is shown in equation (*2*). The pepcarboxykinase reaction was first clearly defined by Utter and Kurahashi (22, 23), who showed that the enzyme from bird liver catalyzed the reaction shown in equation (*3*).

$$\text{PEP} + CO_2 \longrightarrow \text{OAA} + P_i \qquad (2)$$

$$\text{PEP} + CO_2 + \text{GDP (or IDP)} \rightleftharpoons \text{OAA} + \text{GTP (or ITP)} \qquad (3)$$

(PEP, phosphoenolpyruvate; GDP, guanosine diphosphate; IDP, inosine diphosphate; GTP, guanosine triphosphate; ITP, inosine triphosphate; P_i, inorganic phosphate.)

The present work has shown that the pepcarboxykinase of higher plants uses ADP rather than GDP.

MATERIALS AND METHODS

ENZYME PREPARATIONS: Most of the plant materials were purchased from local groceries. Exceptions were lupine, pea seed, wheat germ and corn. Lupine seeds, supplied by Dr. Eric E. Conn, were germinated as described elsewhere (10). At 5 to 8 days the cotyledons were removed, washed and treated as described below. Pea seed acetone powder was furnished by Dr. Helen Stafford. Spinach leaf acetone powder was donated by Dr. R. H. Nieman. Wheat germ S-50 was supplied by General Mills. Corn seeds (Yellow Dent, Variety WF 9 XM 14), a gift from Dr. Richard H. Hageman, were germinated according to his directions in glass casserole trays on acid-washed sand, with 10^{-4} M $CaCl_2$ added to the casserole dish till the solution just covered the sand. The seeds were put on paper towels placed on top of the sand. The casserole dishes were covered with parafilm paper and placed in the dark at room temperature. After 3 days the parafilm paper was removed, and after one more day the seedlings were collected and washed.

In general, the enzyme preparations used were made by blending the washed plant material in a Waring blendor at moderate speeds with enough 0.001 M phosphate buffer of pH 7.4 to give a smooth homogenate. The mixture was strained through two layers of cheesecloth and centrifuged at 0° C in a Servall refrigerated centrifuge at 500 × g for 5 minutes. The sediment was discarded. Ammonium sulfate was added to the supernatant with constant stirring until the solution was three-fourths saturated. After 10 to 15 minutes, the suspension was centrifuged at 0° C for 10 to 15 minutes at 18,000 × g. The precipitate obtained was suspended in cold, distilled water and clarified by centrifugation. The clear supernatant was dialyzed for 3 to 4 hours against several changes of distilled water. The dialysate was clarified by centrifugation and the clear protein solution was lyophilized. The lyophilized powder was the source of the enzyme extract. All operations were carried out in the cold at 4 to 8° C.

FRACTIONATION OF PARTICULATES AND CYTOPLASM: Spinach particulates and cytoplasm for the experiments described in table VIII, were prepared as follows. Spinach leaves were homogenized in 0.35 M NaCl as described by Arnon et al (1, 2). All operations were carried out at 0 to 8° C. The large particles and debris were removed by straining through cheesecloth and centrifuging at 500 × g for one min-

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ute. The supernatant solution was then centrifuged for 30 minutes at 18,000 × g. The supernatant solution obtained from this second centrifugation was the source of the cytoplasmic protein, which was precipitated by ammonium sulfate and lyophilized after salt removal as described in the general procedure. The sediment was washed once by resuspending in 0.35 M NaCl and centrifuging for 20 minutes at 18,000 × g. The precipitate was suspended in cold distilled water and lyophilized. An extract of the powder is referred to as "total particulates." To obtain "intact chloroplasts" and "small particulates," the procedure was the same as that used for preparing total particulates, except that the first centrifugation was carried out at 300 × g, and the suspension was then centrifuged at 1200 × g for 10 minutes, to give a preparation consisting mainly of intact chloroplasts. By final centrifugation for 30 minutes at 18,000 × g, a sediment of smaller particles was obtained. Washing and lyophilization was carried out as for the total particulate fractions, and protein solutions were finally made from the dry powders for the experiments given in table VIII. The "washed chloroplasts" and "washed small particulates" were prepared in the same way except that each fraction was washed three times by suspension and recentrifugation from 0.35 M NaCl before the lyophilization procedure.

The separation of cauliflower mitochondria was carried out as described by Laties (13). The mitochondrial sediment and the supernatant solution from the first high speed sedimentation were treated in an analogous manner to the spinach fractions. That is, the mitochondria were suspended in water and lyophilized. From the dry powder, the soluble protein was extracted to give the solution used in the experiments. The protein from the supernatant was precipitated by addition of $(NH_4)_2SO_4$ to 75 % saturation. The precipitate was redissolved in H_2O and salts were removed by dialysis, as described in the general procedure.

Pea mitochondria were prepared from whole 4-day seedlings ground in 0.2 M sucrose - 0.015 M phosphate of pH 7.0, as described by Price and Thimann (14). The protein of the supernatant from the first high speed centrifugation was recovered by the same procedure as that described for the cauliflower preparations. A protein extract was also prepared from the mitochondria by the same procedure as described for cauliflower.

Reagents: The sodium salts of adenosine diphosphate (ADP), adenosine triphosphate (ATP), guanosine triphosphate (GTP) and inosine diphosphate (IDP) were obtained from Pabst. Crystalline ATP free of other non-adenine nucleotides was used in later experiments and found to give exactly the same results as the amorphous ATP salts. The nucleotides were neutralized with NaOH prior to use. Inosine triphosphate (ITP) was the barium salt from Sigma and was prepared for use as described by Tchen and Vennesland (19) except that NaOH was used for neutralization. Phosphoenolpyruvate (PEP) was obtained from two sources. The Ag-Ba double salt of PEP, a gift from Dr. John Graves, was converted to the neutral Na salt (19). The PEP-tricyclohexylamine salt was purchased from the California Foundation for Biochemical Research. Both preparations of PEP gave similar results in the system employed. Collidine was purchased from Eastman Company. OAA was prepared from the sodium salt of the diethyl ester (Practical) by the procedure of Heidelberger (9). The OAA was neutralized with NaOH just prior to use. DPNH was used as the salt of tris (hydroxymethyl) amino methane, and was a gift from Dr. F. A. Loewus. Protein was determined by the optical method of Warburg and Christian (11, 26).

Procedures and Results

The Exchange Reaction: The pepcarboxykinase reaction was assayed by determination of the exchange of $C^{14}O_2$ into OAA in the presence of ATP (25). The mechanism of this "exchange" is understood as a cycling process, i.e, ATP reacts wtih OAA to give PEP, CO_2 and ADP. The PEP and ADP may then react back with labeled CO_2 to form carboxy-labeled OAA. The reaction has the advantage of high sensitivity as attested by positive results obtained when the reaction products couldn't be detected by chemical analysis.

The experimental procedure involved incubation of the extract to be tested with OAA and $C^{14}O_2$,[4] in the presence and absence of ATP (25). The reaction was stopped by addition of 2 ml of 50 % citric acid to the 10 ml of reaction mixture. The $C^{14}O_2$ was flushed out. Traces of $C^{14}O_2$ were washed out with a stream of unlabelled CO_2, the flushing was repeated, and a sample of the last remaining CO_2 was always collected for radioactivity determination, to ensure that the removal of $C^{14}O_2$ was virtually complete. If the radioactivity determination of the control showed that the $C^{14}O_2$ of the medium had not been entirely removed, the experiment was discarded. After removal of the CO_2 of the medium, the OAA was decarboxylated by addition of 5 ml of aniline citrate reagent. The CO_2 formed was collected in 4 ml of CO_2-free, 1 *N* NaOH. The CO_2 content of an aliquot of this solution was determined manometrically. Then another aliquot (usually 2 ml) of the solution was diluted to 20 ml with CO_2-free water, and sufficient 0.5 M Na_2CO_3 was added to make a total of 250 micromoles of carbonate. The carbonate was precipitated by addition of 0.4 ml of 1 M $BaCl_2$. The $BaCO_3$ was collected by centrifugation and washed with methanol. The washed precipitate was plated, dried and counted at infinite thickness in a gas-flow counter. The counts per minute, corrected for background, were corrected by the known dilution factor to give the counts per minute (cpm) of the collected

[4] The term $C^{14}O_2$ is used here to represent all forms of carbon dioxide and carbonate. During the incubation, the label is present mainly as $HC^{14}O_3^-$, but it may be the unhydrated form, $C^{14}O_2$, which actually participates in the reaction.

CO_2, i.e, of the β-carboxyl group of the OAA. About 50 micromoles of $NaHC^{14}O_3$ containing about 2.5 μc of C^{14} were added to each reaction mixture. This carbonate had a thick sample count in our apparatus of 3×10^6 cpm. All data are given as thick sample cpm determined under identical conditions. The ratio of the specific activity of the β-carboxyl group of OAA to the specific activity of the carbonate of the medium at the beginning of the incubation may be obtained by dividing the figures for "exchange" by the thick sample count of the added $NaHC^{14}O_3$ (3×10^6 cpm).

Table I contains representative results obtained for the exchange reaction with extracts from eighteen different sources. The thick sample count of CO_2 collected from OAA incubated in the absence of ATP was about the same in all experiments, ranging from approximately 200 to 400 cpm. When the incubation mixture contained added ATP, there was a much larger amount of exchange observed in more than half of the cases. The ten sources of the first group in table I all gave a response to ATP of sufficient magnitude to be regarded as unquestionable. Only tobacco leaf and lettuce leaf gave clearly negative responses to ATP. The negative and questionable results are not regarded as proof of the absence of the enzyme, but only as inconclusive. Later experiments showed that extracts of pea seedlings definitely catalyzed the ATP-stimulated exchange, so the results with pea seeds, for example, probably reflect the presence of a small amount of enzyme. The reproducibility of the exchange assay was determined in some instances by as many as five duplicate experiments, and considerable variability in the numerical results was observed. However, the difference between the lowest and highest value of duplicate determinations did not exceed 40 % of the highest figure. It is likely, therefore, that most of the results listed as questionable should be regarded as positive, but the authors prefer to leave the question open in view of possible alternative explanations for small positive effects.

TABLE I

DISTRIBUTION OF THE ATP-STIMULATED EXCHANGE REACTION

ENZYME SOURCE	EXCHANGE ($10^{-2} \times$ CPM)	
	NO ATP	10 MICROMOLES ATP
Corn seedling	2.3	90
Squash fruit	3.1	83
Cauliflower bud	3.6	71
Lupine cotyledon	2.3	70
Turnip	2.4	42
Mustard leaf	2.8	24
Radish leaf	4.1	14
Cabbage leaf	2.9	13
Cucumber fruit	1.8	11
Wheat germ	2.9	10
Pea seed	3.6	6.3
Parsnip root	2.6	4.2
Egg plant	2.2	4.2
Honeydew melon	2.6	4.0
Parsley leaf	2.9	3.6
Spinach leaf	1.8	3.4
Tobacco leaf	1.9	1.9
Lettuce leaf	2.0	1.7

The test system contained 100 micromoles of OAA, 100 micromoles of $MnCl_2$, 50 micromoles of $NaHC^{14}O_3$ (3.0×10^6 cpm), 500 micromoles of collidine buffer of pH 7.0, and enzyme, in a total volume of 10 ml. Incubation was for 30 minutes at room temperature, which was generally 23° C. Except for the case of pea seed and spinach leaf, the enzyme was prepared by suspending 20 to 30 mg of lyophilized powder per ml of deionized water, and centrifuging down the insoluble residue. One ml of the clear supernatant solution was added to the incubation mixture.

The preparation from pea seed was made by fractionation of an aqueous extract of the acetone powder with ammonium sulfate. The protein which precipitated between 25 and 70 % saturation was dissolved in water and dialyzed overnight against 0.005 M collidine buffer of pH 7.4. In the assay 1.5 ml of solution, corresponding to 2 gm of acetone powder, was used. In the case of spinach, the acetone powder was extracted with 0.002 M collidine of pH 7.0. The extract was dialyzed overnight against 0.01 M collidine of pH 6.8, and clarified by centrifugation. One ml of material, corresponding to 50 mg of acetone powder, was used in the assay.

TABLE II

REQUIREMENTS OF THE "BLANK" EXCHANGE REACTION

REACTION SYSTEM	EXCHANGE ($10^{-2} \times$ CPM)
Complete	24
No ATP	2.8
Complete, enzyme heat-inactivated	2.7
No enzyme	2.6
No enzyme, no Mn^{++}	0.4
No enzyme, no OAA	0.2

The reaction mixture and procedure were the same as that used in the experiments of table I. Mustard leaf was the enzyme source; and 13 mg of protein were used in the test system. When OAA was omitted during the incubation, it was added later as a carrier to provide CO_2 during the decarboxylation.

The data in table I shows that definitely positive results were obtained with four of the eight families tested. The reaction occurs in both monocotyledonous and dicotyledonous plants, and active preparations were obtained from fruits, roots, buds, cotyledons, seeds and leaves.

THE "BLANK" EXCHANGE: The results in table I show a small and relatively constant exchange in the absence of ATP, no matter what the enzyme source. Further experimentation showed this reaction to be largely non-enzymatic, and dependent on added Mn^{++}. Table II shows the experimental evidence for this conclusion. The complete system contained all the components of the assay, including ATP. It is clear that the same small exchange was observed if the enzyme was omitted, or heat-inactivated, or if the ATP was omitted. When Mn^{++} was omitted, however, the exchange decreased to about one tenth of the amount observed in the presence of Mn^{++}. It seems reasonable to assume that the Mn^{++}-dependent exchange is a

measure of the reversibility of the non-enzymatic Mn^{++}-catalyzed decarboxylation of OAA. The justification for this assumption is detailed in the following paragraph.

To assess the significance of the "Blank" exchange, one may calculate from the best available data how much $C^{14}O_2$ exchange might be expected to occur if the decarboxylation was a one-step reaction and the exchange represented a simple reversal of this reaction. For the reaction

$$OAA^{=} + H_2O \longrightarrow \text{pyruvate} + HCO_3^-$$

Burton and Krebs (6) have calculated the free energy change at unit activity and 25° C, $\Delta F° = -6.39$ kilocal. From this value, the equilibrium constant, $K = \frac{(HCO_3^-)\ (\text{pyruvate})}{(OAA)}$ may be calculated.

$\Delta F° = -RT \ln K$ (where R is the gas constant and T is the absolute temperature). $K = 4.9 \times 10^4$. For the reaction

$$OAA^{=} \underset{k'}{\overset{k}{\rightleftharpoons}} \text{pyruvate} + HCO_3^-;$$

$$K = \frac{k}{k'} = 4.9 \times 10^4 \text{ moles/L}$$

If the amount of C^{14} entering OAA is represented by x, then

$$\frac{dt}{dx} = k'\ (\text{pyruvate})\ (HCO_3^-)\ \frac{C^{14}}{(HCO_3^-)}$$

[Where $\frac{C^{14}}{(HCO_3^-)}$ is the specific activity of the HCO_3^- at any particular time and the parentheses represent concentrations in moles/L]

This gives equation I:

$$\text{I.}\quad dx = k' \cdot C^{14}\ (\text{pyruvate})\ dt$$

Divide equation I by an equation II:

$$\text{II.}\quad d(\text{pyruvate}) = k(OAA)dt = k[A - (\text{pyruvate})]dt$$

where A is the initial concentration of OAA.

This gives

$$\frac{dx}{d(\text{pyruvate})} = \frac{k'}{k} \cdot C^{14} \cdot \frac{(\text{pyruvate})}{[A - (\text{pyruvate})]}$$

or

$$dx = \frac{C^{14}}{K} \cdot \frac{(\text{pyruvate})}{[A - (\text{pyruvate})]} d(\text{pyruvate})$$

This expression may be integrated to solve for x when any particular fraction of the OAA has been decarboxylated.

For 50 % decarboxylation,

$$\int_0^x dx = \frac{C^{14}}{K} \int_0^{A/2} \frac{(\text{pyruvate})}{[A - (\text{pyruvate})]} d(\text{pyruvate}) + C$$

$$\int_0^{A/2} \frac{(\text{pyruvate})}{[A - (\text{pyruvate})]}$$

$$= \Big|_0^{A/2} [A - (\text{pyruvate})] - A \ln[A - (\text{pyruvate})]$$

$$= A\ (\ln 2 - \tfrac{1}{2})$$

$$C = 0$$

$$x = 0.19 \cdot \frac{C^{14}}{K} \cdot A$$

or

$$\frac{x}{C^{14}} = 0.19 \frac{A}{K}$$

If

$$A = 1 \times 10^{-2}$$

$$\frac{x}{C^{14}} = 3.9 \times 10^{-8}$$

If $C^{14} = 100$, $x = 3.9 \times 10^{-6}$, and is the % of the total C^{14} which will have been introduced into the β-carboxyl group by the time half of the OAA has been decarboxylated. Since the calculation does not take into account the fixed C^{14} which will be lost again by decarboxylation, the above figure is too high. The amount of OAA which was decarboxylated in the blank sample was generally somewhat less than 50 %, in which case the calculated amount of C^{14} fixed will also be high so the statement is justified that *less* than

TABLE III

CHARACTERISTICS OF THE ATP-STIMULATED EXCHANGE REACTION

TIME OF INCUBATION		ENZYME CONC		Mn^{++} CONC		ATP CONC	
TIME	EXCHANGE ($10^{-2} \times$ CPM)	PROTEIN	EXCHANGE ($10^{-2} \times$ CPM)	Mn^{++}	EXCHANGE ($10^{-2} \times$ CPM)	ATP	EXCHANGE ($10^{-2} \times$ CPM)
min		*mg*		*micromoles*		*micromoles*	
5	8	3.9	38	0	0.5	0	3
15	17	7.8	46	5	1.8	5	14
30	28	12.9	170	25	15	10	25
60	72	20.7	390	50	24	25	25
..	..	...	...	100	43	50	30
..	..	...	...	...	..	100	24

Except for the variant under examination, the conditions were the same as described in the legend for table I. Each reaction mixture contained 3.5 mg of protein from turnip. The data are not sufficiently accurate to warrant the conclusion that the departures from linearity, with time and with protein concentration, are real.

TABLE IV

COMPARISON OF Mg^{++} AND Mn^{++}

ENZYME SOURCE	EXCHANGE ($10^{-2} \times$ CPM)		
	NO METAL	Mg^{++}	Mn^{++}
Turnip	2.0	7.6	47
Cauliflower bud	2.2	6.1	68
Squash	4.8	19	63
Lupine cotyledon	1.8	52	69

Conditions as in table I. One hundred micromoles of the chloride salt were added.

3.9×10^{-6} % of the total C^{14} would be introduced into the β-carboxyl group.

The calculated value may now be compared with the value actually observed in the blank. The thick sample count of the CO_2 from the 50 micromoles of OAA left at the end of the reaction was generally about 3×10^2 per minute. The thick sample count of the 50 micromoles of $HC^{14}O_3^-$ present in the medium initially was 3×10^6 counts per minute. The ratio $3 \times 10^2/3 \times 10^6 = 1 \times 10^{-4}$, or 1×10^{-2} percent of the C^{14} added was actually observed to be fixed. This is three orders of magnitude greater than the calculated value. Though the free energy data and the experimental data are subject to considerable error, these errors are probably not large enough to account for the large discrepancy between the experimental result and the calculated result. This discrepancy must therefore be regarded as evidence that the mechanism assumed for the exchange reaction is incorrect. The most likely alternative is that the reaction proceeds in two steps, with an enol of pyruvate formed as an intermediate. It seems probable that the enzyme-catalyzed decarboxylation and the non-enzymatic, Mn^{++}-catalyzed decarboxylation are alike in this respect (8, 18).

PROPERTIES OF THE ATP-DEPENDENT EXCHANGE REACTION: A series of experiments were carried out to determine the dependence of the exchange reaction on the components of the system. The affects of varying time of incubation, amount of protein, Mn^{++} concentration, and ATP concentration are shown in table III. When Mg^{++} was substituted for Mn^{++}, the effect varied with the enzyme source tested (table IV).

NUCLEOTIDE SPECIFICITY: After extensive purification of the pepcarboxykinase from avian liver, Utter and his collaborators were able to show that the reaction utilized ATP only indirectly (12, 22, 23). The natural substrate appeared to be GTP, though ITP was also active. Similarly, the pepcarboxykinase of lamb liver was shown by Bandurski and Lipmann (5) to use ITP in preference to ATP. The present studies included an examination of the nucleotide specificity of the ATP-dependent exchange catalyzed by enzyme preparations from several of the plant sources. Preparations from turnip were tested most extensively, with ATP, ITP and GTP. Representative preparations from five other sources were examined with ATP and ITP. The results are summarized in table V. In each experiment, the blank determination and the determination with added nucleotides were carried out with the same enzyme under strictly comparable conditions. The major conclusion was that in every one of the cases examined, ATP was much more effective than ITP. ATP was also considerably more effective than GTP with the turnip preparation. The previously reported finding (19) that ITP was as effective as ATP with wheat germ could not be confirmed. This may have been due to the fact that the enzyme preparations were very impure. It is possible that the discrepancy may be due to a transphosphorylation brought about by a trace of adenine nucleotide present in either the ITP or the enzyme preparation. In any event, it appears that ATP is most likely to be the natural substrate for the pepcarboxykinase from higher plants.

DISTRIBUTION OF PEPCARBOXYLASE: The spectrophotometric test described by Tchen and Vennesland (19) was used to test the various enzyme preparations for the presence of pepcarboxylase. This test is applicable only in the presence of malic dehydrogenase. It is based on the oxidation of DPNH by OAA formed from added PEP, the oxidation being measured by following the decrease in optical density at 340 mμ. The enzyme solutions were tested first to ensure that DPNH was rapidly oxidized by added OAA, but not by added pyruvate. All the enzyme preparations tested fulfilled this criterion. The effect of added PEP on the oxidation of DPNH was then determined. The enzyme preparations usually caused an oxidation of DPNH in the absence of added PEP. When this DPNH oxidation was rapid, the assay procedure was less reliable, but the use of careful controls often made it possible to conclude that the addition of PEP definitely increased the rate of oxidation of DPNH. In addition to spinach leaf and

TABLE V

NUCLEOTIDE SPECIFICITY OF EXCHANGE REACTION

ENZYME SOURCE	EXCHANGE ($10^{-2} \times$ CPM)			
	NO NUCLEOTIDE	ITP	ATP	GTP
Turnip	2.4	3.0	42	...
"	...	...	171	22
Corn seedling	2.3	6.3	90	...
Squash	3.1	9.0	83	...
Cauliflower bud	4.5	7.7	73	...
Lupine cotyledon	2.2	3.8	43	...
Wheat germ	2.9	2.2	10	...

The test system was the same as that of table I. Nucleotides were added as specified in the amount of 10 micromoles per 10 ml reaction mixture. The two experiments with turnip were performed with different enzyme preparations.

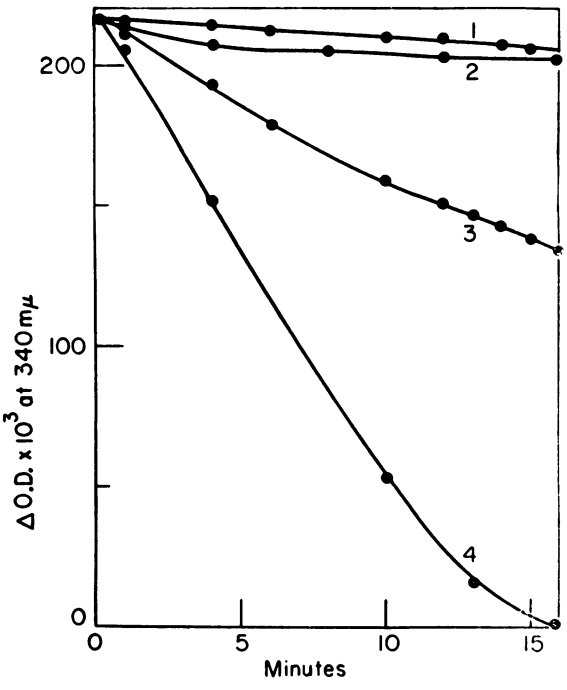

FIG. 1. The reaction mixtures contained 60 micromoles of phosphate buffer of pH 7.0, 10 micromoles of $MgCl_2$, 0.117 micromoles of DPNH, and other addenda as indicated, in a total volume of 3 ml. Each mixture contained 0.1 ml of the same extract from pea seed as was used in the experiment of table I. The reaction mixture was placed in cuvettes of 1 cm light path and the optical density at 340 mμ was measured with a Beckman spectrophotometer. The readings plotted on the graph have been corrected for absorption of all components other than the DPNH. Curve 1, no added substrate; curve 2, 2 micromoles of PGA and 30 micromoles of NaF; curve 3, 2 micromoles of PGA; curve 4, 2 micromoles of PEP. The curve for PEP and NaF could almost be superimposed on that for PEP without NaF.

wheat germ, which have previously been shown to contain pepcarboxylase, the following enzyme sources gave clearly positive tests: pea seed, parsnip root, cauliflower buds, cabbage leaf, turnips, lupine cotyledon, mustard leaf, and squash. With other sources tested, results were questionable or negative, but these negative results should not be regarded as definitive proof of the absence of the enzyme. In some of the cases where added PEP caused an oxidation of DPNH, added phosphoglyceric acid (PGA) was also tested, and found to be effective, but to a lesser extent than PEP. The oxidation of DPNH by PGA under these circumstances could be inhibited by NaF, but this reagent did not affect the oxidation of DPNH by PEP. These phenomena are similar to those already reported in detail for spinach and wheat germ. The inhibition by F^- is readily explained by the action of this inhibitor on enolase. A typical set of experiments with pea seed is shown in figure 1.

The DPNH oxidase activity of the extract from pea seed was smaller than that generally encountered in the various enzyme sources tested. Other data are not given in detail because of the general similarity of the results to those previously documented.

THE FORMATION OF C^{14}-OAA FROM PEP AND ADP: Pepcarboxylase will cause the formation of β-carboxy-labeled OAA (C^{14}-OAA) from PEP and $C^{14}O_2$, but pepcarboxykinase can cause C^{14}-OAA formation only if ADP is added with PEP. The dependence of the reaction on addition of both ADP and PEP can best be demonstrated in the absence of pepcarboxylase. Experiments which illustrate this are shown in table VI. The enzyme preparations used had been shown to be free of pepcarboxylase. The data in the table also show that pyruvate alone was inactive as a substrate, and that IDP could not be substituted for ADP.

Since no cycling is necessary when OAA is formed directly from PEP, CO_2 and ADP, the data of table VI should give a more realistic measure of the rate at which OAA may be formed by the pepcarboxykinase

TABLE VI

SYNTHESIS OF OAA FROM PEP AND $C^{14}O_2$

ENZYME SOURCE	ADDITIONS IN MICROMOLES	C^{14}-OAA (10^{-2} × CPM)
Turnip root	100 PEP, 50 ADP	236
" "	100 PEP	0.3
" "	50 ADP	0.3
" "	100 Pyruvate	0.2
Corn seedling	50 PEP, 100 ADP	140
" "	50 PEP, 100 IDP	3.4
" "	50 PEP	0.6

The reaction mixtures contained 500 micromoles of collidine buffer of pH 7.0, 100 micromoles of $MnCl_2$, $C^{14}O_2$ as in the "exchange" assay, 1 ml of enzyme, and other components as specified, in a final volume of 10 ml. Incubation was for 30 minutes at room temperature. After the reaction had been stopped by the addition of citric acid, 50 micromoles of OAA were added as carrier. The decarboxylation of OAA and the determination of the radioactivity of the CO_2 were then carried out exactly as in the "exchange" experiments previously described. The results, given in counts per minute, corrected for background, are comparable with those of the previous tables. The enzyme preparation from corn seedling contained 9.5 mg of protein per ml. The preparation from white turnips contained 7.3 mg of protein per ml. This preparation had been partially purified for the ability to catalyze the ATP-stimulated exchange reaction. The turnips were homogenized with phosphate buffer, the solids were removed by centrifugation and the activity was precipitated from the extract by addition of $(NH_4)_2SO_4$ to 60% saturation. The protein was dissolved in water, and the salt was removed by dialysis. A precipitate which formed when the solution was heated to 50° C for 5 minutes was removed by centrifugation. The pH was adjusted to 5.2 with acetic acid and a suspension of calcium phosphate gel was added (0.2 mg per mg protein. The gel was removed and the supernatant was fractionated with $(NH_4)_2SO_4$. The most active fraction was obtained between 35 and 50% saturation. It was dialyzed to remove salt, and lyophilized. The purification as measured by the ATP-stimulated exchange was 10-fold over the first extract, per mg protein.

reaction than any of the data obtained by measurement of the ATP-stimulated exchange. The latter test has the advantage, however, of being qualitatively more specific. Thus, pepcarboxylase will give no ATP-stimulated exchange reaction, but it will cause OAA formation from PEP and CO_2 alone, without added ADP. Under these circumstances, it is obviously difficult to determine a small amount of pepcarboxykinase. For this reason mainly, the ATP-stimulated exchange test was selected for use in the broad survey of the distribution of pepcarboxykinase. It is necessary to keep in mind, however, that even though pepcarboxylase alone can cause no ATP-stimulated exchange whatever, it can cause an increase in the magnitude of the exchange test observed if pepcarboxykinase is present. Any quantitative evaluation of the ATP-stimulated exchange test therefore requires consideration of the possible effect of the pepcarboxylase which may be present. It is possible, for example, that the relatively large stimulatory effect of Mg^{++} observed in the ATP-stimulated exchange catalyzed by a lupine cotyledon preparation (table IV) may be due to a relatively large amount of pepcarboxylase present, since pepcarboxylase appears to respond to Mg^{++} better than does pepcarboxykinase in those cases where the enzymes have been tested separately (3).

RESULTS WITH SPINACH: Spinach leaf had been shown in previous work by others (4) to give no ATP-stimulated exchange reaction, but to be an excellent source of pepcarboxylase. Because of the large amount of enzyme present and the absence of pepcarboxykinase activity, spinach appeared to be well suited for a study of the possible localization of pepcarboxylase. Previous work with spinach had all been done with extracts of acetone powders. Since acetone treatment disrupts particulate structures, it could not be employed in the localization studies. Solubles and particulates were therefore separated by centrifugation, as described in the section on Materials, and protein-containing extracts from the various fractions were tested for catalysis of labeled OAA formation from PEP and $C^{14}O_2$ in the presence and absence of ADP. Note that in all cases a protein solution was used for the experiment. No chlorophyll was present in the incubation mixtures.

The results given in table VII show that pepcarboxylase is associated with the particulate fraction and that it is not removed by repeated washing of the particulates in the suspending medium used, though the enzyme is readily solubilized after lyophilization. The small particulate fraction which consisted of mitochondria and chloroplast fragments, was more active per mg protein than the intact chloroplast fraction, but the intact chloroplasts contained considerable activity. The question of whether the enzyme is really present in the chloroplast or in smaller particulates, or in both, should probably be left open, however, since it is difficult to determine the extent to which the chloroplasts are contaminated by mitochondria (which have been shown to be present in leaves (15)), and since the smaller particulate fraction certainly contained a high proportion of chloroplast fragments. The washing procedure removed protein from both fractions, but most of the enzyme activity was retained, with the result that the activity per mg protein was considerably increased by washing.

TABLE VII

INTRACELLULAR LOCALIZATION OF ENZYMES IN SPINACH LEAF

PREPARATION TESTED	MG PROTEIN	C^{14}-OAA ($10^{-2} \times$ CPM)		SPECIFIC ACTIVITY *	
		50 MICROMOLES PEP	50 MICROMOLES PEP, 100 MICROMOLES ADP	50 MICROMOLES PEP	50 MICROMOLES PEP, 100 MICROMOLES ADP
Total particulates	4.7	38	100	8.1	21
Cytoplasm	17.7	0.05	0.09	0.003	0.005
Intact chloroplasts	5.8	5	20	0.9	3.4
Small particulates	5.6	54	147	9.7	26
Washed chloroplasts	1.7	29	59	17	35
Washed small particulates	1.9	98	230	52	121

The procedure was the same as that described for table IV.

* Specific activity $= \frac{10^{-2} \times \text{cpm}}{\text{mg protein}}$

The surprising fact which emerged from these experiments was that ADP caused a considerable stimulation of the β-carboxyl-labelled OAA formation. This was not expected because of previous failure to detect pepcarboxykinase in spinach. The simplest interpretation of the unexpected ADP effect was that pepcarboxykinase was indeed present, and that previous failures to detect it were due to the inactivation of the enzyme by the different preparative procedures previously employed. The spinach particulates were therefore tested for their ability to cause an ATP-stimulated exchange reaction. The results were negative. Because of the anomalous nature of these results, the experiments were repeated many times. The ADP effect was readily duplicable. Two- to four-fold stimulation of C^{14}-OAA formation was obtained with fresh particulate preparations, but no ATP-stimulated exchange reaction was observed. Table VIII shows the results of experiments performed to test the variation of the effect with the amount of ADP added, and the effect of IDP. It is apparent that IDP is as effective as ADP, in contrast to the results obtained with the other plant sources. Elucidation of the nature of the ADP effect observed with spinach requires further experimentation, with a tentative conclusion that the effect cannot be attributed to a pepcarboxykinase of the sort demonstrated and studied in other plant sources.

Localization Studies with Cauliflower and Pea: A few preliminary experiments were carried out with cauliflower bud homogenate and with pea seedlings, to see whether the enzymes could be localized in the soluble or particulate fractions. Both of these sources contained both pepcarboxylase and pepcarboxykinase activity. The question of localization is of interest, because a mixture of the enzymes would behave like an ATP-ase. That is, the net effect of the two enzymes acting together (with a small amount of either PEP or OAA) would be to convert any ATP which was present to ADP and inorganic phosphate. It would be interesting to know whether the cell actually employs such a mechanism for hydrolyzing ATP.

Efforts to show a completely separate localization of the two enzymes in solubles and particulates of cauliflower buds and pea seedlings were not successful. The results obtained with the fractions from cauliflower are given in table IX. Each fraction was tested for its ability to cause labelled OAA formation from PEP alone, from PEP and ADP, and from OAA in the presence of ATP. The latter tests were carried out as described for the assay procedure of table I. The other experiments were carried out as described for the experiments of table VIII. The data of table IX shows that no clear-cut separation of the enzymes was achieved by the centrifugation procedures employed, and that the cytoplasmic protein appears to be richer in both enzymes. In the case of pea seedling, there was a higher ATP-stimulated exchange in the particulate protein fraction than in the soluble protein. Thus, the standard "exchange" assay gave a value of 8.4×10^2 cpm for 6.5 mg of mitochondrial protein, and 4.6×10^2 cpm for the same amount of cytoplasmic protein. The latter result is close to the blank level, but is not clearly negative.

Discussion

One of the main questions pertinent to function is the problem of the rate at which the carboxylases operate to form OAA. A measure of this rate is provided by the experiments in which the enzyme preparation is incubated with PEP and $C^{14}O_2$, with or without ADP. The data given as counts per minute of C^{14}-OAA can be converted to micromoles of OAA formed per unit time for a given amount of enzyme preparation by multiplying the cpm for C^{14}-OAA by $50/3 \times 10^6$. (50 micromoles of bicarbonate with a thick sample count of 3×10^6 were present in the incubation mixture, and the CO_2 was collected with 50 micromoles of carrier OAA. One may assume that the amount of OAA formed is inappreciable in relation to the amount of carrier added. Though this is not strictly correct, the error introduced by the assumption is probably no greater than the errors of the measurements.) Thus, for corn seedling (table VI), $14{,}000 \times 50/3 \times 10^6 = 0.23$ micromoles of C^{14}-OAA was formed in the incubation period of 30 minutes. The present studies were not planned to obtain accurate rate measurements, and the figures should only be regarded as approximate. There is also no assurance that test conditions were optimal, or that the preparation of the extracts did not entail a loss of activity.

Carboxylase and carboxykinase may both be regarded as enzymes catalyzing steps in the conversion of carbohydrate to malate. The well-known crassulacean acid metabolism of the succulents has been shown to consist of the production of malic acid at night with a diminution of carbohydrate followed by a loss of malate in daylight with an increase of carbohydrate (20). It has been suggested that this diurnal fluctuation is not confined to the succulents, but is widespread throughout higher plants, the difference between succulents and non-succulents being quantitative rather than qualitative (21). Saltman et al (16) have identified OAA as the probable first product of dark CO_2-fixation in leaves of *Bryophyllum calycinum*, and have demonstrated the presence of pepcarboxylase in leaf extracts. The present work shows that both pepcarboxylase and pepcarboxykinase are widely distributed.

Table VIII

ADP Effect with Spinach Particulates

Expt. no.	Nucleotide	C^{14}-OAA ($10^{-2} \times$ cpm)
1	.....	500
	10 micromoles ADP	630
	50 micromoles ADP	980
	100 micromoles ADP	1050
2	.....	210
	100 micromoles ADP	570
	100 micromoles IDP	490

The reaction mixtures and procedures were the same as those described for table VI, except that 50 micromoles of PEP were added in every case and the nucleotides were added as indicated. In experiment 1, each reaction mixture contained 3.4 mg of protein from small particulates. In experiment 2, each reaction mixture contained 3.8 mg of protein from another preparation of small particulates. The small particulates were prepared as described for the experiments of table VII.

Table IX

Results with Cauliflower Bud Homogenate

Enzyme source	Additions in micromoles	C^{14}-OAA ($10^{-2} \times$ cpm)
Mitochondria	100 PEP	20
	100 PEP, 50 ADP	64
	100 OAA, 10 ATP	6
Cytoplasm	100 PEP	186
	100 PEP, 50 ADP	254
	100 OAA, 10 ATP	19

The experiments with mitochondria were carried out with 3.8 mg of protein, and those with cytoplasm were carried out with 7.3 mg of protein. The reaction mixtures and procedures for the experiments with PEP alone and with PEP and ADP were the same as described in the legend for table VI. The experiments with OAA and ATP were carried out as described for table I.

The OAA formed by carboxylation would not be expected to accumulate. One of the most active enzymes in plant tissues is malic dehydrogenase, and any OAA formed would be immediately reduced to malate if reduced diphosphopyridine nucleotide (DPNH) were available. An unpublished study has been made in our laboratory by A. Magaldi, of the relative rates of oxidation of DPNH by various oxidants in the presence of acetone powder extracts from a variety of plant sources. The results with OAA, calculated as micromoles DPNH oxidized per hour per mg dry weight of the extract, may be listed as follows: pea shoot, 90; pea root 210; pea seeds 28, 36; parsley leaf 31, 30, 11.5; cress shoot, 101; carrot root 11; wheat seedling root, 31; and wheat germ, 220. Oxidation of DPNH by pyruvate never approached such activities, and the oxidation of DPNH by hydroxypyruvate in the presence of leaf extracts, which is a rapid reaction (17), was never more than 15 % as rapid as the oxidation by OAA.

In connection with the spectrophotometric assays of pepcarboxylase described in the present paper, measurements were always made of the rate of oxidation of DPNH by OAA and by pyruvate in the presence of the various extracts tested. These tests showed that OAA always oxidized DPNH at a rate much more than sufficient to account for the reaction observed with added PEP, whereas the oxidation of DPNH by pyruvate was always too slow to account for the PEP effect observed. The results all confirmed the generalizations made from the earlier observations of Magaldi, that malic dehydrogenase activity is always high, and lactic dehydrogenase activity is low or absent. In many instances, where DPNH is oxidized by addition of pyruvate, it is possible that the pyruvate is first decarboxylated to acetaldehyde which then acts as oxidant for DPNH. (The relatively high lactic dehydrogenase content of potato appears to offer an exception to the general rule (17).

The data available on enzyme activities therefore suggest that in many, if not all, plant tissues, malate should be regarded as the natural end-product of the anaerobic phase of carbohydrate breakdown, analogous to lactate in muscle and ethanol in yeast.[5] (This does not imply that lactate and ethanol formation have no functional significance in higher plants, but only that malate formation is more important quantitatively in most cases. Lactate formation appears to be of minor significance, with ethanol formation occupying an intermediate position.) Since many plants contain a malic enzyme (24) in addition to pepcarboxylase and pepcarboxykinase, the interplay of all three CO_2-fixing reactions must be taken into account in any description of the course of molecular events within the intact tissue. The three CO_2-fixing enzymes must have different functional roles. It has been pointed out previously (19) that pepcarboxykinase can cause a conversion of OAA to PEP and so may act in the synthesis of carbohydrate from organic acids, whereas pepcarboxylase cannot function in this way. Furthermore, since the direction of the pepcarboxykinase reaction can be determined by the concentration levels of CO_2, ADP, and ATP, it is not difficult to visualize a means whereby the photosynthetic production of ATP and utilization of CO_2 could lead to malate disappearance, whereas increase in CO_2 and conversion of ATP and ADP would lead to malate formation. The pepcarboxylase reaction, on the other hand, could account for the conversion of carbohydrate to malate at low CO_2 tensions, and would not respond to changes in the concentration levels of ATP and ADP. Nevertheless, the fact that these various enzymes have different functional potentialities does not completely define their relations to each other, and the manner in which their action is integrated within the cell is a subject worthy of further investigation.

[5] If malate formation does not occur anaerobically, but requires O_2, then the formation of PEP cannot occur over the classical path from fructose-1,6-diphosphate by way of the aldolase and triose-phosphate dehydrogenase reactions, which would provide adequate amounts of DPNH.

Summary

Assay procedures have been described for detecting pepcarboxylase and pepcarboxykinase in the tissues of higher plants, with special attention to the problems encountered in assay when the two enzymes are present in the same extract. Both enzymes were shown to be widely distributed in plant tissues. They frequently occur in the same source. The pepcarboxykinase of higher plants is specific for ATP rather than other nucleotides. Anomalous results with spinach particulates showed that OAA formation from PEP was enhanced by ADP and IDP, though other tests for pepcarboxykinase were negative. The pepcarboxylase of spinach was localized in particulates. In cauliflower buds and pea seedlings, both the mitochondrial and the cytoplasmic proteins appeared to contain both enzymes, though in different amounts.

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GROWTH REGULATORS AND FLOWERING. I. SURVEY METHODS[1,2]

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Recent investigations have shown that applied auxin may have either a positive or a negative effect upon the flowering of certain plants (e.g., 3, 7, 9, 14, 16, 22, 23, 26, 29, 32, 35, 36). Growth regulators other than auxin (e.g., maleic hydrazide (18, 30), triiodobenzoic acid (12), gibberellin (21, 37), cobaltous ion (24), etc.) have also been shown to influence the flowering process in a number of plants. On the basis of the auxin effects, theories of the mechanism of floral induction have been proposed (1, 6, 10, 19, 20, 24, 25, 31), and additional insight into the partial processes of induction has been gained (27, 33).

Specifically, the present work was carried out to answer the following four questions: 1) Does a particular growth regulator influence flowering? 2) Which phase of the flowering process does it influence? 3) Will effects upon flowering illuminate the flowering mechanism? 4) Will effects upon flowering illuminate the mechanism of action of a given growth regulator upon growth?

Seven compounds were chosen as representatives of growth regulator types presently considered to be of importance: maleic hydrazide (MH), 2,2-dichloro-

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