FIXATION OF CARBON DIOXIDE IN PARTICULATE PREPARATIONS FROM BARLEY ROOTS [1]

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Non-photosynthetic fixation of CO_2 has been shown to occur in a wide range of tissues and more recently investigations have been focussed on the mechanisms of the carboxylation reactions involved. Mazelis and Vennesland (19) have summarized the 3 different carbon fixing processes as a) the malic enzyme reaction (20), b) the phosphoenolpyruvate carboxykinase (or pepcarboxykinase) reaction (29, 30), and c) the phosphoenolpyruvate carboxylase (or pepcarboxylase) reaction (3). The widespread occurrence of these 3 enzymes in higher plants has been shown (19, 31), and their possible physiological functions have been discussed (19, 27, 33).

Fixation of CO_2 by roots has been reported by various workers (7, 11, 12, 18, 21), however few investigations have been made on the carboxylation reactions involved. As ion absorption influences the level of both fixation and labelled malate formation (12, 22), and as it has been suggested that the mitochondria may act as ion carriers in ion absorption (23), it was of interest to determine whether the mitochondria may act as a site for CO_2 fixation in roots and also to investigate the mechanism whereby fixation is accomplished. Our findings have been reported briefly elsewhere (8, 35), and the effect of salts on the CO_2 fixation in the particulate preparations is reported in another paper (36). Independent evidence of pepcarboxylase activity in extracts of Snap bean roots has also been reported (10).

MATERIAL AND METHODS

The particulate preparations were isolated from excised roots of 6-day-old barley seedlings. Initially the variety California Mariot was used; in later experiments Tennessee Winter. The seedlings were grown in a dilute nutrient solution in the dark (13). The excised roots were centrifuged for 5 minutes at 65 × G to remove the excess water and then chilled at 2.5° C for at least 1 hour. Initially the excised roots were ground with sand in a mortar, but this was unsatisfactory owing to the very low CO_2 fixation activity of the preparations (0.1 % incorporation of the total carbon-14 added). The most active preparations were obtained by blending roots in a Waring blendor for 1 minute in a root to solution ratio of 1 : 1 chilled medium of 0.5 M sucrose, 0.001 M versene, and 0.05 M potassium phosphate at pH 7.6 to 8.0. In some experiments particles were isolated in 0.5 M sucrose solution buffered with 0.05 M TRIS[3]; but these preparations had less than 30 % normal fixation activity. The homogenate was filtered through 4 layers of cheesecloth, and the filtrate was centrifuged at −5° C in a Servall centrifuge at 1,200 × G for 10 minutes to give the low speed residue consisting of cell nuclei, cell walls, larger particles and isolated cells. The supernatant was centrifuged at 16,000 × G for 20 minutes. The pellet obtained was suspended in 0.4 M sucrose solution and recentrifuged at the same speed for another 10 minutes to give the high speed residue. The pellet was ground with a glass homogenizer, and 0.4 M sucrose solution was added to make a final particulate preparation containing 3 parts solution to 1 part pellet.

The labelled potassium bicarbonate solution was prepared by liberating CO_2 from carbon-14 labelled barium carbonate with acid and absorbing the labelled CO_2 in KOH solution. The specific activity of the $KHC^{14}O_3$ solution was 2.5×10^5 cts per sec per micromole. The tricyclohexylamine salt of PEP was obtained from the California Foundation for Biochemical Research; DPNH was generously donated by Dr. E. E. Conn, TPNH by Dr. R. Whatley, and phosphoserine by Dr. J. A. Gladner of the National Institute of Health, Bethesda.

The particles were exposed to $C^{14}O_2$ in Stanley manometer flasks (26) at 25° C. After an equilibration period the labelled bicarbonate was added to the reaction medium from the fixed side arm. At the end of the exposure period the reaction was terminated by the addition of 0.5 ml 2 *N* H_2SO_4 from 1 of the movable side arms, and the other side arm containing 0.5 ml 5 *N* KOH was opened to allow the absorption of excess $C^{14}O_2$ for a period of 15 minutes. Any residual labelled CO_2 was displaced by bubbling inert CO_2 through the reaction medium for an additional 15 minutes. In experiments where DPNH was present in the reaction medium, the DPNH was added after the equilibration period from the side arm

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[3] The following abbreviations will be used: TRIS, trishydroxymethylaminomethane; PEP, phosphoenolpyruvic acid; DPN and DPNH, oxidized and reduced diphosphopyridine nucleotide; AMP, adenosine monophosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; IDP, inosine diphosphate; ITP, inosine triphosphate; GDP, guanosine diphosphate; UDP, uridine diphosphate.

which normally contained KOH; in these experiments the excess $C^{14}O_2$ was removed solely by bubbling with inert CO_2. Blank experiments, using heat-inactivated particulate preparations, showed that the removal of the labelled CO_2 was complete by this method.

The contents of the flask were poured into centrifuge tubes and centrifuged for 5 minutes in a clinical centrifuge; no attempt was made to obtain a quantitative recovery. Aliquots of the supernatant and the residue fractions were plated out on copper discs and the activities of the samples were determined using a windowless flow counter. Knowing the dilution factors the total amount of $C^{14}O_2$ fixation in the preparation was estimated. In all cases, the layer of the plated sample was so thin that self absorption was negligible when calculated from the data of Libby (18).

Carbon-14 activity in the organic acids was determined by ether extraction of aliquots of the supernatant fraction followed by silica gel chromatography after the method of Bulen et al (6). The 2,4-diphenylhydrazones of the keto acids were prepared according to the method of Virtanen et al (32) from aliquots of the supernatant fraction and the carbon-14 activity in the hydrazones was measured. Identification of the labelled keto acids was made by 1-dimensional paper chromatography of the hydrazones with hydrazones prepared from known standard keto acids using 0.2 M phosphate buffer at pH 8.0 followed by radioautography. Radioautographs were also prepared from aliquots of the supernatant fractions; initially 2-dimensional paper chromatograms were run using phenol: water and butanol: proprionic acid: water as the 2 solvents as recommended by Benson et al (5). In later experiments 1-dimensional chromatograms were made using the solvent system isopropanol: ammonia: water (70: 10: 20) and in this system the solvent was allowed to traverse the paper and drip from the end for a period of 3 days. This solvent not only gave a better separation of the phosphorylated compounds, but also the spots were unaffected by the presence of the salts in the reaction medium. Isopropanol: ammonia: water system had been used for some of the hydrazones. Protein hydrolysis of the residue fraction was carried out using either 2 *N* NaOH or 6 *N* HCl in sealed tubes at 117° C for a period of 12 hours, and aliquots of the hydrolysates were run on 2-dimensional paper chromatograms.

TABLE I

OXIDATION OF ORGANIC ACIDS BY BARLEY ROOT PARTICLES

SUBSTRATE ADDED	O_2 UPTAKE μl/MG N/HR
None	0
α-ketoglutarate	53
Succinate	53
Citrate	34
Malate	56
Glutamate	45
Pyruvate trace of malate	63

Reaction mixture: 1 ml particles containing 1 to 2 mg nitrogen, 60 μM substrate, 120 μM sucrose, 30 μM glucose, 9 μM AMP, 9 μM magnesium chloride, 30 μM sodium fluoride, 45 μM phosphate buffer at pH 7.4, 1 mg cytochrome c and 0.10 mg DPN in a total volume of 3 ml.

Experimental conditions: Temperature 25° C. O_2 uptake measured manometrically.

TABLE II

$C^{14}O_2$ FIXATION IN BARLEY ROOT PARTICLES IN THE PRESENCE OF PHOSPHOENOLPYRUVATE AND PYRUVATE *

A. DISTRIBUTION IN FRACTIONS

SUBSTRATE	FRACTIONS		
	SUPERNATANT	RESIDUE	TOTAL
1. Pyruvate (sodium salt)	1.1	1.0	2.1
2. Phosphoenolpyruvate (cyclohexamine salt)	12.0	2.6	14.6

B. EFFECT OF pH

SUBSTRATE	pH		
	4.8	5.6	7.4
1. Pyruvate (sodium salt)	1.4	1.5	0.3
2. Phosphoenolpyruvate (cyclohexamine salt)	2.0	2.6	12.0

* Activity as % of total carbon-14 added.

Reaction mixture: 1 ml particles containing 1.30 mg nitrogen, 60 μM substrate, 4.5 μM magnesium chloride, 0.6 μM manganese chloride, 90 μM phosphate buffer at 1. pH 5.6, 2. pH 7.4, and 1 μM potassium bicarbonate-^{14}C with 2.5 $\times$ 10^5 counts/sec carbon-14 activity in a total volume of 3.3 ml. In addition: 1. 9 μM ATP; 2. 9 μM AMP.

Experimental conditions: Temperature 25° C; exposure period 1 hr.; reaction terminated by 0.5 ml 2 *N* sulphuric acid; excess $C^{14}O_2$ removed by bubbling with inert CO_2 for 15 minutes.

RESULTS

Initial experiments were carried out to determine the ability of the particulate preparations to utilize various organic acids as substrates. The rates of O_2 uptake in the presence of added organic acids are given in table I. The results indicated that the particulate preparations were able to utilize these organic acids as substrates and that they probably possessed the enzymes necessary for the functioning of the Krebs tricarboxylic acid cycle.

$C^{14}O_2$ fixation was studied in the presence of either added PEP or added pyruvate and the results are shown in table II. The presence of PEP greatly increased the level of CO_2 fixation as compared to the fixation with pyruvate, and this increase was mainly in the supernatant fraction. The effect of

added pyruvate to the medium as compared to no added substrate was variable, in some cases giving no increase in the level of CO_2 fixation and in other cases a slight stimulation. Throughout the present investigation either pyruvate or PEP was added to the reaction medium, and these conditions for CO_2 fixation were termed the pyruvate and PEP systems respectively.

The effect of pH was studied in the 2 systems and CO_2 fixation was measured at pH values 4.8, 5.6 and 7.4 (table II). The results showed that the optimum pH requirements for the 2 systems differed; in the presence of pyruvate the highest activity was at 5.6, whereas in the presence of PEP it was 7.4. The rate of fixation by the 2 systems was measured and it was found that fixation was practically completed in 20 minutes in the supernatant in the PEP system, while in the presence of pyruvate the fixation continued to increase with time over a period of 1 hour (table III). Both systems were heat-labile and no CO_2 fixation took place in heat inactivated preparations. The PEP system in the particulate preparations retained its activity during storage and could be kept in a refrigerator for as long as a month without much loss of activity, whereas the pyruvate system lost its activity overnight.

Radioautographs of the pyruvate system supernatant showed activity in asparagine, aspartate and glutamate with traces of activity in malate. In corresponding radioautographs of the PEP system most of the activity was distributed between aspartate, glutamate, malate and phosphoserine; the distribution of activity in these compounds varied with pH with a marked increase in phosphoserine at pH 5.6 compared to pH 7.4. Hydrolysates of the residue fractions showed a wide range of amino acids and radioautographs showed labeling into most of the amino acids present. No attempt was made to identify these amino acids. Radioautographs of the 2,4-diphenylhydrazones showed that in both systems labelled oxalacetate and pyruvate were formed. A large amount of labelled oxalacetate along with traces of labelled glyoxalate appeared in the presence of PEP.

It was found that in the PEP system approximately 80 % of the fixation occurred in the hydrazones as compared to about 22 % in the pyruvate system. These results, together with the radioautograph data indicated that conditions were limiting for the further metabolism of the labelled oxalacetate. Preliminary spectrophotometric measurements of the oxidation of DPNH demonstrated that the particulate preparations contained malic dehydrogenase. The addition of DPNH to the PEP system resulted not only in an increase in the total CO_2 fixation, but also in the carbon-14 activity of the hydrazone fraction and the ether soluble fraction, in fumarate and succinate, particularly in malate. The addition of malate to the reaction medium, both in the absence or presence of DPNH, stimulated fixation. Measurements of the carbon-14 activity in the 2,4-diphenylhydrazones showed that even in the presence of added DPNH considerable activity still remained in the keto acids. Although increasing the concentration of added DPNH in the PEP + DPNH treatment resulted in a higher level of fixation in the hydrazone fraction, the presence of added malate decreased fixation. The results of these experiments are summarized in table IV.

The labelled malate was degradated to determine the distribution of the carbon-14 within the acid using a similar procedure to Jacobson (12). Trial runs showed that approximately 75 % of the malate was decarboxylated and determinations of the activity of the CO_2 produced and the remaining activity in the reaction medium in the form of pyruvate and non-decarboxylated malate indicated that the activity in malate was equally divided between 2 carboxyl groups.

The effect on CO_2 fixation of other organic acids was investigated; addition of oxalacetate (18 micromoles (μM)) to the medium greatly decreased fixation both in the presence or absence of DPNH. The presence of isocitrate (60 μM) in the PEP + DPNH system resulted in a decrease in fixation and paper chromatographic analysis of the 2,4-diphenylhydrazones of the keto acids showed an increase in the formation of labelled glyoxalate under these conditions. When the preparations were exposed to added isocitrate in the presence of TPNH, labelled glycollate was formed.

In the pyruvate system the effect of adding malate, or malate and DPNH, differed from the PEP system in that the CO_2 fixation was reduced rather than increased (see table V). In order to stimulate malic enzyme activity TPNH was added, however in both the PEP system and the pyruvate system the presence of TPNH resulted in a reduction in the CO_2 fixation (table V). The level of fixation in the pyruvate system being so low the small reduction caused by TPNH may not be significant. Fixation in the pyruvate system in this experiment was carried out

TABLE III

$C^{14}O_2$ FIXATION IN BARLEY ROOT PARTICLES IN RELATION TO TIME

TIME (MN)	CARBON-14 ACTIVITY (COUNTS/SEC).			
	PEP SYSTEM		PYRUVATE SYSTEM	
	SUPERNATANT	RESIDUE	SUPERNATANT	RESIDUE
2	1,830	34	189	14
4	3,580	156	301	32
6	5,230	184	343	37
8	6,730	266	437	64
10	7,850	318	371	51
20	12,350	790	437	103
30	11,600	1,044	579	200
60	15,300	1,420	1,140	300

Reaction mixture: 1 ml particles containing 1.10 mg nitrogen, otherwise as in table II.

Experimental conditions: See table II, except for different exposure periods.

TABLE IV

$C^{14}O_2$ FIXATION IN BARLEY ROOT PARTICLES BY THE PEP SYSTEM IN THE PRESENCE OF ADDED DPNH *

TREATMENT	PEP	PEP + MALATE	PEP + DPNH	PEP + × 2 DPNH	PEP + DPNH + MALATE	PEP + × 2 DPNH + MALATE
Total fixation	13.08	28.27	28.27	29.76	30.77	24.58
Total ether extract	0.86	1.64	6.04	7.73	12.99	10.56
Fraction						
Acetate	0	0.01	0.06	0.06	0.12	0.07
Pyruvate	0	0.01	0.03	0.02	0.03	0.03
Fumarate	0.06	0.14	0.88	1.22	2.16	1.58
Succinate	0.09	0.22	0.33	0.13	0.25	0.20
Pyrrolidone carboxylate	0.31	0.38	0.30	0.21	0.34	0.23
Oxalate	0.04	0.08	0.05	0.11	0.10	0.30
Malate	0.29	0.66	4.13	5.81	9.69	7.74
Isocitrate	0.03	0.10	0.18	0.11	0.25	0.28
Citrate	0.03	0.06	0.06	0.05	0.04	0.10
Tartrate	0.01	0.02	0.02	0.01	0.01	0.03
Fixation in ether extract expressed as % total carbon-14 fixed.	6.6	5.8	21.4	26.0	42.2	42.5
Hydrazones	10.98	19.90	15.09	11.80	14.10	11.33
Fixation in hydrazones expressed as % total carbon-14 fixed.	79.6	70.9	53.2	39.6	45.8	46.1

* Activity as % of total carbon-14 added.

Reaction mixture: 1 ml particles containing 1.23 mg nitrogen, 1 μM PEP (cyclohexamine salt), 3 μM magnesium chloride, 1.2 μM manganese chloride, 9 μM AMP, 90 μM phosphate buffer at pH 7.4 and 1 μM potassium bicarbonate-^{14}C with 2.5 × 10^5 counts/sec carbon-14 activity in a total volume of 4.3 ml. 1.6 μM DPNH, 3.2 μM DPNH and 18 μM malate added as indicated.

Experimental conditions: See table II.

TABLE V

$C^{14}O_2$ FIXATION IN BARLEY ROOT PARTICLES BY THE PEP AND PYRUVATE SYSTEMS IN THE PRESENCE OF ADDED DPNH, MALATE AND TPNH *

	TREATMENT	FRACTION			
		SUPERNATANT	RESIDUE	TOTAL	ETHER EXTRACT
A.	Pyruvate	0.27	0.10	0.37	0 20
	Pyruvate + malate	0.25	0.08	0.33	0.24
	Pyruvate + malate + DPNH	0.23	0.07	0.30	0.21
B.	PEP	47.00	0.29	47.30	7.10
	PEP + malate	62.10	0.25	62.40	6.30
	PEP + malate + DPNH	72.90	0.50	73.40	32.70
C.	PEP	39.16	0.56	39.72	2.21
	PEP + TPNH	32.83	0.69	33.52	2.45
	PEP + TPNH + malate	26.01	0.55	26.56	4.99
D.	Pyruvate	0.73	0.14	0.87	...
	Pyruvate + TPNH	0.62	0.12	0.74	...

Reaction mixture: A. 1 ml particles containing 1.30 mg nitrogen, 60 μM sodium pyruvate, 3 μM magnesium chloride, 1.2 μM manganese chloride, 9 μM ATP, 90 μM phosphate buffer at pH 5.6 and 1 μM potassium bicarbonate-^{14}C with 2.5 × 10^5 counts/sec carbon-14 activity in a total volume of 4.5 ml. 1.6 μM DPNH and 18μM malate added as indicated.

B. 1 ml particles containing 1.41 mg nitrogen, otherwise as in table IV.

C. 1 ml particles containing 1.01 mg nitrogen. 1.9 μM of TPNH added as indicated, otherwise as in table IV.

D. 1 ml particles containing 1.56 mg nitrogen, in a total volume of 3.9 ml. 1.9 μM of TPN added as indicated, otherwise as in A.

Experimental conditions: See table II.

at pH 7.4 as this is the optimum pH for malic enzyme activity.

During the course of the investigation a study of the cofactor requirements was made for the pyruvate system and the PEP systems, both in the presence and absence of DPNH. The results of these experiments involving various cofactors are summarized in table VI. The presence of both magnesium and manganese ions was required in all these systems. The nucleotide requirements differed in that AMP stimulated fixation in the PEP and PEP + DPNH systems, whereas it had no effect on the pyruvate system; ATP stimulated fixation in the pyruvate system but markedly reduced fixation in the presence of PEP. The addition of other nucleotides—ADP, ITP, IDP, GDP and UDP—in place of AMP all resulted in a lower level of CO_2 fixation in the PEP system. The carboxylation of PEP was partially inhibited in the presence of 0.05 μM *p*-chloromercuri-benzoate and completely inhibited with 0.5 μM of the inhibitor. The use of TRIS as a buffer in the reaction medium resulted in a marked reduction in the level of fixation in both the PEP in the absence of DPNH and pyruvate systems; in the pyruvate system the level of fixation in TRIS was compared with that using phosphate buffer at pH 7.4. However, the presence of TRIS instead of phosphate in the PEP + DPNH system apparently increased fixation. The effect of flushing the reaction medium for 15 minutes with cylinder nitrogen to produce low O_2 tensions reduced the level of CO_2 fixation in both the PEP and pyruvate systems.

TABLE VI

COFACTOR REQUIREMENTS IN $C^{14}O_2$ FIXATION IN BARLEY ROOT PARTICLES

MEDIUM	SYSTEM		
	PEP	PEP + DPNH	PYRUVATE
	Activity as % fixation in complete medium		
Complete	100	100	100
$-MnCl_2$	72	35	96
$-MgCl_2$	89	7	88
−AMP	84	86	100
−AMP + ATP	37	60	118
−AMP + ADP	51	55	...
−AMP + ITP	16	59	...
−AMP + IDP	...	95	...
−AMP + GDP	81	73	...
−AMP + UDP	81	79	...
+PCMB 10^{-4} M	88	...	...
+PCMB 10^{-3} M	0	...	...
$-PO_4$ buffer + TRIS Cl_2	54	120	67
Complete N_2 gas phase	51	...	64

Reaction mixture: Complete medium; 1 ml particles containing 1 to 2 mg nitrogen, 3 μM magnesium chloride, 1.2 μM manganese chloride, 9 μM AMP, 90 μM phosphate buffer at pH 7.4 (PEP and PEP + DPNH systems) and at pH 5.6 (Pyruvate system), and 1 μM potassium bicarbonate-^{14}C with 2.5×10^5 counts/sec carbon-14 activity in a total volume of 4.5 ml. Also 1 μM PEP (cyclohexamine salt) in PEP and PEP + DPNH systems. 1.6 μM DPNH in PEP + DPNH system. 60 μM sodium pyruvate in pyruvate systems. Further additions as indicated: 9 μM ATP, 9 μM ADP, 9 μM ITP, 9 μM IDP, 9 μM GDP, 9 μM UDP, 0.05 μM *p*-chloromercuribenzoate (PCMB), 0.5 μM PCMB, 30 μM TRIS chloride at pH 7.4, nitrogen gas phase-flask containing reaction medium flushed with cylinder nitrogen for 15 minutes before exposure to $C^{14}O_2$.

Experimental conditions: See table II.

DISCUSSION

The results demonstrated that the particulate preparations from barley roots were capable of CO_2 fixation; preliminary experiments with other fractions of the root extract, namely the low speed residue and the high speed supernatant (see Methods), also gave $C^{14}O_2$ fixation, so that it appeared unlikely that the carboxylation reactions were confined to any 1 fraction of the root extract. However as no steps were taken to purify the fractions, and no examination of the fractions was made, this point has not been conclusively demonstrated.

The results indicated that there were at least 2 carboxylating systems responsible for CO_2 fixation in the preparations, and that pepcarboxylase activity could be extremely high. Similar experiments with lupine root particles (unpublished data) gave the same pattern, with low fixation in the presence of pyruvate and a marked increase in fixation in the presence of PEP. The results for the barley particulate preparations showed that the amount of fixation varied considerably with different preparations from about 12 % to over 70 % of the total carbon-14 added. Presumably these differences were due to slight variations in the preparation of the material. It was also observed that the amount of fixation depended on both the concentration of labelled bicarbonate and on the amount of particles present; fixation increasing proportionately with higher concentrations of either bicarbonate or particles. For convenience the quantity of particles used in each experiment was expressed in terms of the nitrogen content.

The fixation of CO_2 in the pyruvate system was probably similar to the low levels of fixation found by others (3, 10) in the presence of pyruvate, although their exposure times were considerably shorter, and in the present investigation the fixation in the pyruvate system continued to increase with time over a period of 1 hour. The higher activity at pH 5.6 and the decrease of fixation in the presence of added TPNH indicated that this might not be the malic enzyme reaction. No appreciable quantity of labelled malate was formed even in the presence of added DPNH in the pyruvate system. The relatively high incorporation of the fixed carbon-14 activity by this

system into the amino acids, obtained from both the supernatant and the hydrolysate of the residue fraction, indicated that the labelled compounds formed in the presence of pyruvate underwent transamination and were utilized in protein synthesis.

The CO_2 fixation in the PEP system was essentially similar to the pepcarboxylase reaction (3) apart from the presence of AMP as a cofactor. The addition of other phosphate nucleotides ADP (19, 27), IDP (29), GDP (19) and UDP (29) all decreased fixation; this reduction may have been due to either pyruvic kinase activity, which would result in the breakdown of PEP to pyruvate, or pepcarboxykinase activity, which would result in the reversible carboxylation of PEP. We have been unable to offer any satisfactory explanation for the stimulation due to the presence of AMP. Evdence for pepcarboxykinase activity was found in small amounts of labelled PEP on the chromatograms. This could be derived by decarboxylation of α carboxyl labelled oxalacetate. Labelling of oxalacetate by pepcarboxylase has been shown to be almost entirely into the β carboxyl (3, 34), however our degradative studies of labelled malate showed equal labelling of the α and β carboxyl groups. A similar distribution of carbon-14 activity in malate has been found in other investigations with root material (2, 12). Furthermore, our preliminary experiments indicated an abundant labelling of the α position as well as the β position in oxalacetate.

Labelled glyoxalate formation was stimulated in the presence of added isocitrate indicating isocitritase activity (25); the addition of isocitrate to the medium resulted in the increased carbon-14 activity in the glyoxalate. The further addition of TPNH to this system resulted in the reduction of the labelled glyoxalate to give labelled glycollate (14). These results indicated that in our preparations there was the possible functioning of the glyoxalate bypass (16) and that α labelled malate might be formed by the action of malate synthetase (15). Further experiments are necessary to determine the importance of these findings.

The apparent inorganic phosphate requirement in both the PEP and pyruvate systems has also been observed in pepcarboxylase activity in wheat germ (27) although they were unable to find any exchange between inorganic phosphate and the phosphate moiety of PEP; in our experiments the use of TRIS as a buffer stimulated fixation in the presence of DPNH contrasting with the experiments without added DPNH. We have not investigated the mechanism of the formation of labelled phosphoserine in our preparations apart from observing its occurrence during fixation by the PEP system. No labelled glyceric acid (9, 24) was observed on our chromatograms, and spraying with ninhydrin showed that the carbon-14 activity was present in the amino carboxyl group of the phosphoserine.

Inhibition of pepcarboxylase activity by *p*-chloromercuribenzoate has also been reported for wheat germ preparations (27), and the reduction in CO_2 fixation under low O_2 tensions (28, 34) indicated that fixation in both the pyruvate and PEP systems depended on the normal functioning of the Krebs tricarboxylic acid cycle.

In conclusion our results have indicated that there are several carboxylating systems present in the particulate preparations of barley roots. The occurrence of an active pepcarboxylase system in roots provides 1 mechanism whereby roots may utilize both the endogenous CO_2 and that present in the soil in organic acid synthesis. The irreversible formation of oxalacetate takes place at low concentrations of CO_2 in contrast to the pepcarboxykinase and malic enzyme reactions which require much higher levels of CO_2 (28). The reduction of oxalacetate by malic dehydrogenase would result in an accumulation of malate, which has been shown to contain most of the carbon-14 activity in fixation studies with intact roots. The possible functioning of the glyoxalate by-pass would provide not only a means for malate formation under low O_2 tensions, but also a pathway for the interconversion of fat and carbohydrate (15). Products of fixation in the pyruvate system can undergo transamination and be synthesized into protein, whereas the conversion of oxalacetate to PEP by pepcarboxykinate can bring about the synthesis of carbohydrates from organic acids.

Summary

Investigations on CO_2 fixation showed that the particulate preparation from barley roots provided an active system for fixation of CO_2 through carboxylation of PEP. In the presence of pyruvate a low level of fixation was found. A study of the conditions and cofactor requirements was made for fixation by the 2 systems. The pepcarboxylase activity could be linked to malic dehydrogenase activity by the addition of DPNH to stimulate the formation of labelled malate. Evidence obtained from radioautographs indicated the presence of pepcarboxykinase activity and the possible functioning of the glyoxalate by-pass. The physiological significance of these reactions was briefly discussed.

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