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THE CONTROL OF PLANT GLUTAMATE DEHYDROGENASE BY PYRIDOXAL-5′-PHOSPHATE

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Key Word Index—Glutamate dehydrogenase; pyridoxal-5′-phosphate; pyridoxamine-5′-phosphate; *Lemna minor*; *Haplopappus gracilis*; *Pisum sativum*; control of glutamate synthesis.

Abstract—The proposition that the nitrogen status of a plant is reflected by the ratio pyridoxal phosphate to pyridoxamine phosphate and that this ratio exerts a controlling influence on plant metabolism has been examined. The ratio pyridoxal phosphate to pyridoxamine phosphate has been shown to increase during nitrogen starvation. The inhibition of glutamate dehydrogenase by pyridoxal phosphate has been examined and the kinetics of inhibition are discussed in relation to the proposed control of metabolism.

INTRODUCTION

PYRIDOXAL-5′-phosphate is required for a number of enzyme reactions involving transaminations and amino acid decarboxylations in which it participates through formation of a Schiff base.[1] In addition, it is also required in a number of cases where Schiff base formation is not involved—glycogen phosphorylase,[2] tryptophanase,[3] aspartate-β-decarboxylase.[4]

The possibility that pyridoxal phosphate is formed from pyridoxamine phosphate by a specific transaminase was considered by Beechey and Happold[5] and they suggested that control of this transaminase could affect the whole pattern of amino acid metabolism of the cell. Subsequent work has failed to demonstrate the presence of a pyridoxamine-5′-phosphate transaminase but the existence of a pyridoxamine-5′-phosphate oxidase has been established in both bacteria[6] and animals.[7,8] Furthermore, an enzyme (peroxidase) which catalyses the destruction of pyridoxal-5′-phosphate in the presence of amino acids and manganous ions has been demonstrated in extracts of pea seedlings.[9] These enzymes provide an alternative route for changing the concentrations of pyridoxal phosphate and

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pyridoxamine phosphate and re-establish the potential for metabolic control suggested by Beechey and Happold.

This possibility has been strengthened by the finding that pyridoxal phosphate inhibits a number of purified enzymes including glutamate dehydrogenase,[10-12] hexokinase,[13] 6-phosphogluconate dehydrogenase,[14] phosphofructokinase,[15,16] aldolase,[17] pyruvate kinase,[18] malate dehydrogenase[19] and fructose-1,6-diphosphatase.[20]

A number of kinetic studies of glutamate dehydrogenase from higher plants[21-24] have failed to establish a scheme for metabolic control of this important enzyme. Consequently, we have examined the effect of pyridoxal-5'-phosphate on glutamate dehydrogenase and have attempted to relate this to the general proposition that the nitrogen status of the plant is reflected by the ratio pyridoxamine phosphate/pyridoxal phosphate.[25]

RESULTS

Effect of pyridoxal-5'-phosphate and pyridoxamine-5'-phosphate on glutamate dehydrogenase of pea mitochondria

The partially purified enzyme (Table 1) was found to be inactivated by low concentrations of pyridoxal-5'-phosphate in both directions of assay at pH 7·5 whereas pyridoxamine-5'-phosphate had no effect. The loss of enzyme activity was time-dependent (Fig. 1) and proportional to the concentration of pyridoxal phosphate (Fig. 2).

The degree of inhibition by pyridoxal phosphate was dependent upon the pH of the incubation mixture; the extent of inhibition increasing with pH to a maximum at pH 7·5 then rapidly falling so that at pH 8·0 the inhibition was negligible (Fig. 3).

Reversibility of the inhibition produced by pyridoxal-5'-phosphate

When the enzyme was incubated with pyridoxal phosphate at pH 7·5 to produce maximum inhibition, then transferred to pH 8·0 before assay, the inhibition decreased in a time dependent way (Fig. 4). When the enzyme was assayed in the direction of reductive amination, the inhibitory effect of pyridoxal phosphate was substantially reversed by the addition of 2-oxoglutarate, NH_4Cl or NADH, but not by glutamate or NAD (Fig. 5A). When the enzyme was assayed in the direction of oxidative deaminiation, the inhibitory effect of pyridoxal phosphate was partially reversed by glutamate but not by NAD or aspartate (Fig. 5B). 2-Oxoglutarate, NADH and NH_4Cl added their own inhibitory effects. The effect of NH_4Cl concentration on the reversal of inhibition produced by pyridoxal phosphate is shown in Fig. 6.

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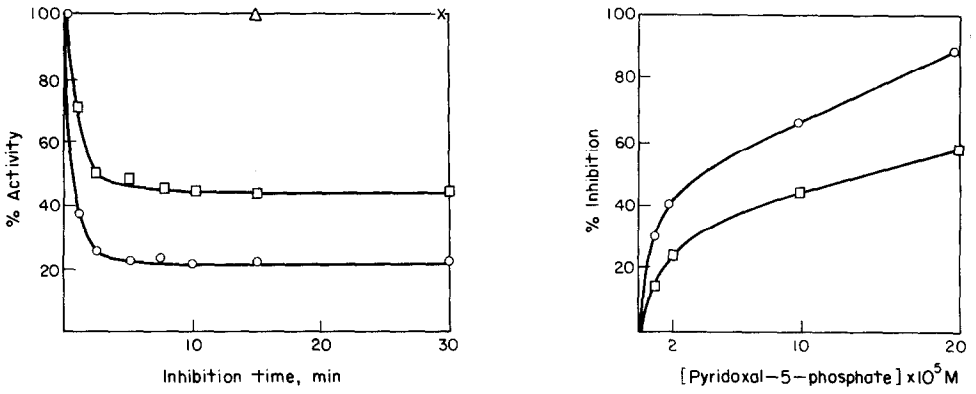


FIG. 1. (Left) THE EFFECT OF INCUBATION TIME ON THE INHIBITION OF GLUTAMATE DEHYDROGENASE BY PYRIDOXAL-5'-PHOSPHATE.

Enzyme (0·12 mg protein for the amination assay, 0·24 mg for deaminating assay) was incubated with buffer (TES pH 7·5 50 mM) and pyridoxal phosphate (0·2 mM) at 30°. At times indicated the substrates were added and the rate of reaction measured at E_{340} nm as described in the text. △——△ Control for amination reaction; ×——× control for deamination reaction; ○——○ amination reaction; 2-oxoglutarate (2 mM), NH_4^+ (0·13 M), NADH (0·2 mM); □——□ deamination reaction; glutamate (13·3 mM), NAD^+ (0·4 mM).

FIG. 2. (Right) EFFECT OF PYRIDOXAL-5'-PHOSPHATE CONCENTRATION ON THE ACTIVITY OF GLUTAMATE DEHYDROGENASE.

Enzyme was incubated with pyridoxal phosphate in TES buffer (pH 7·5, 50 mM) for 15 min at 30° before adding substrates and measuring activity at E_{340}. Assay conditions as in Fig. 1. ○——○ Amination reaction; □——□ deamination reaction.

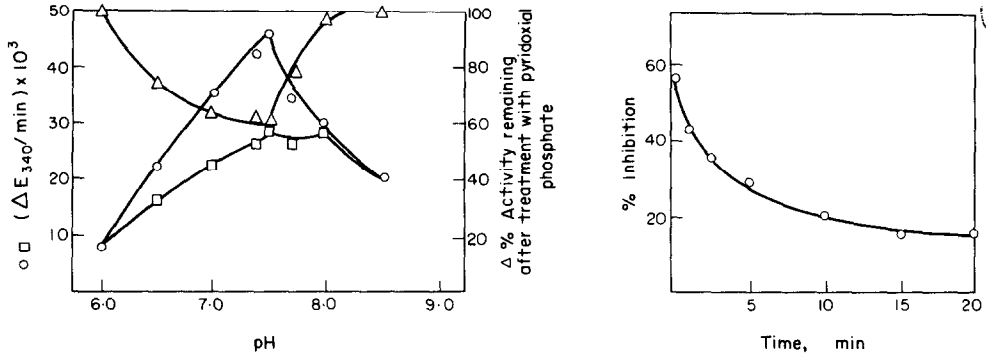


FIG. 3. (Left) EFFECT OF pH ON THE INHIBITION OF GLUTAMATE DEHYDROGENASE BY PYRIDOXAL-5'-PHOSPHATE.

Enzyme was incubated with pyridoxal phosphate (0·02 mM) and buffer for 15 min at 30° before adding substrates and measuring the activity in the direction of reductive amination. Assay conditions: 2-oxoglutarate (2 mM) NH_4Cl (0·13 M), NADH (0·2 mM) enzyme (0·10 mg protein), pyridoxal phosphate (0·02 mM) and buffer (50 mM) in a final volume of 3 ml. Buffers used: Potassium phosphate pH 6, 6·5, 7·0; TES buffer pH 7·4, 7·5; Barbital pH 7·7, 8·0, 8·5; ○——○ control; □——□ enzyme inhibited by pyridoxal phosphate (0·02 mM); △——△ % activity remaining in inhibited enzyme.

FIG. 4. (Right) EFFECT OF TIME AND pH ON THE INHIBITION OF GLUTAMATE DEHYDROGENASE BY PYRIDOXAL-5'-PHOSPHATE.

Enzyme pre-incubated with pyrodoxal phosphate (0·04 mM) in TES buffer pH 7·5 (20 mM) for 20 min at 30°. Aliquots were reincubated in barbital buffer (0·04 mM) containing pyridoxal phosphate (40 μM) for the indicated time intervals before adding substrates and measuring activity in the direction of reductive amination. Assay conditions: 2-oxoglutarate (2 mM), NH_4Cl (0·13 M), NADH (0·2 mM) enzyme (0·15 protein) and barbital buffer (pH 8·0, 50 mM) in a final volume of 3 ml.

TABLE 1. PURIFICATION OF MITOCHONDRIAL GLUTAMATE DEHYDROGENASE FROM PEA EPICOTYLS

Fraction	Volume (ml)	Units (ml)	Proteins (mg/ml)	Specific activity	Yield (%)	Purification
Crude extract	15	0·37	7·2	0·044	100	—
After centrifuging at 48000 *g*	13	0·296	3·5	0·084	81	1·8
After G-25	17	0·214	2·35	0·091	76	2
After heating at 70°	16	0·205	0·76	0·27	68	6
After calcium phosphate gel	10	0·190	0·14	1·34	40	30
After Sephadex G-200						
Fraction 24	2	0·075	0·05	1·50	3	34
Fraction 25	2	0·214	0·04	5·35	7	121
Fraction 26	2	0·204	0·025	8·15	8·5	185
Fraction 27	2	0·205	0·02	10·24	8·6	232
Fraction 28	2	0·111	0·025	4·42	4·6	100
Fraction 29	2	0·086	0·03	2·86	3·6	65
Combined fractions 25–28	8	0·193	0·029	6·65	32	150

The possibility that the aldehyde group of pyridoxal-5-phosphate competes with the carbonyl group of 2-oxoglutarate was examined by measuring the rate of reductive animation at various concentrations of 2-oxoglutarate and pyridoxal phosphate. The results plotted in a standard way suggested non-competitive inhibition.

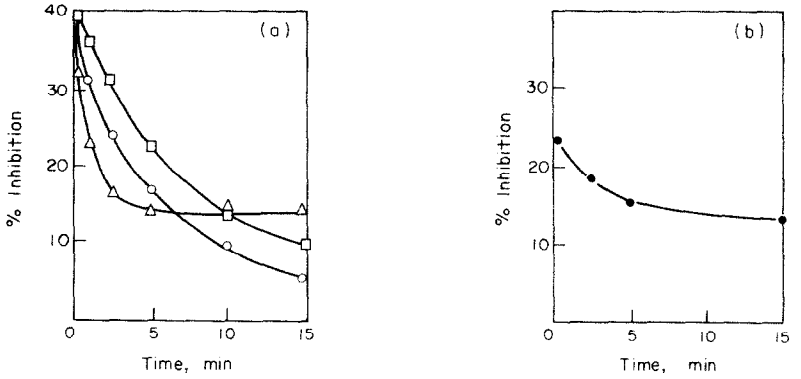


FIG. 5. REVERSIBILITY OF THE PYRIDOXAL-5'-PHOSPHATE INHIBITION OF GLUTAMATE DEHYDROGENASE BY SUBSTRATES.

Enzyme was incubated with pyridoxal-5-phosphate (0·02 mM) in TES buffer (pH 7·5, 50 mM) for 20 min at 30°. A single substrate was then added (concentration given below) and at the indicated time intervals the remaining substrates were added and the enzyme assayed. (A) In the direction of reductive amination. (B) In the direction of oxidative deamination. (a) Assay for reductive amination: 2-oxoglutarate (2 mM), NADH (0·2 mM) NH_4Cl (0·13 M), enzyme (0·12 mg protein) and TES buffer (pH 7·5, 50 mM); ○——○ incubation with NH_4Cl (0·13 M); □——□ incubation with NADH (0·2 mM); △——△ incubation with 2 oxoglutarate (2 mM). (b) Assay for oxidative deamination: Glutamate (13·3 mM) NAD^+ (0·2 mM), enzyme (0·36 mg protein) and TES buffer (pH 7·5, 50 mM); ●——● incubation with glutamate (13·3 mM).

Effect of pyridoxal-5'-phosphate on the activity of other enzymes present in pea extracts

The effect of 0·2 mM pyridoxal phosphate on the activity of a number of other oxidoreductases, malic enzyme, PEP carboxylase and phenylalanine ammonia lyase in pea extracts was tested (Table 2). Glucose-6-P dehydrogenase, 6-phosphogluconate dehydrogenase, alcohol dehydrogenase and lactate dehydrogenase were all inhibited to some extent

TABLE 2. EFFECT OF PYRIDOXAL-5'-PHOSPHATE ON THE ACTIVITY OF SEVERAL ENZYMES PRESENT IN A CRUDE EXTRACT OF PEA EPICOTYLS

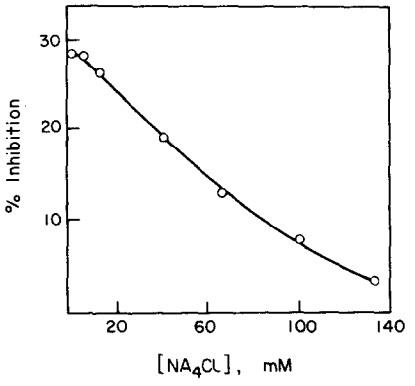
Enzyme	E.C. no.	Control	E_{340}/min/ml + PLP	Inhibition (%)
Glucose-6-P dehydrogenase	1.1.1.49	0·54	0·31	43
6-Phospho-gluconate dehydrogenase	1.1.1.44	0·58	0·43	26
Isocitrate dehydrogenase	1.1.1.42	0·23	0·23	None
Malate dehydrogenase	1.1.1.37	8·8	8·8	None
Shikimate dehydrogenase	1.1.1.25	0·23	0·23	None
Alcohol dehydrogenase	1.1.1.1	0·18	0·15	18
Lactate dehydrogenase	1.1.1.27	0·23	0·16	30
Glutamate dehydrogenase	1.4.1.2	0·24	0·12	50
Malic enzyme	1.1.1.40	0·31	0·31	None
PEP carboxylase	4.1.1.31	0·27	0·27	None
Phenylalanine ammonia lyase	4.3.1.5	0·07	0·07*	None

Pea epicotyl extract was incubated with potassium phosphate buffer (pH 7·5, 50 mM) in the presence and absence of pyridoxal phosphate (0·2 mM) before being assayed for the enzymes listed below. For details see text.
* Assayed at $E_{290\ nm}$.

but not as much as the glutamate enzyme. None of the other enzymes examined were affected.

Effect of nitrogen supply on the concentration of pyridoxal-5'-phosphate and pyridoxamine-5'-phosphate in Lemna minor *and* Haplopappus gracilis

Lemna minor was grown under sterile conditions as previously described in work from this laboratory.[26,27] Subcultures were made from the main culture on media containing



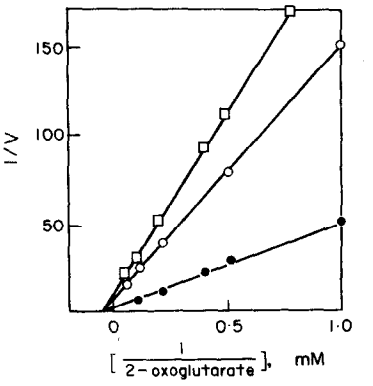


FIG. 6. (Left) EFFECT OF NH_4Cl CONCENTRATION ON THE REVERSAL OF GLUTAMATE DEHYDROGENASE INHIBITION BY PYRIDOXAL PHOSPHATE.
(Enzyme was incubated with pyridoxal phosphate (0·02 mM) in TES buffer (pH 7·5, 50 mM) for 20 min at 30°, then NH_4Cl was added to give various concentrations and after incubating for a further 15 min, the remaining substrates were added and the enzyme assayed in the direction of reductive amination. Assay conditions as in Fig. 5A.

FIG. 7. (Right) INHIBITION OF GLUTAMATE DEHYDROGENASE BY PYRIDOXAL PHOSPHATE.
Enzyme was incubated with pyridoxal phosphate in TES buffer (pH 7·5, 50 mM) for 15 min, at 30° before adding substrates and measuring activity at E_{340}. Assay conditions as in Fig. 1. ●——● Control; ○——○ pyridoxal phosphate (0·1 mM); □——□ pyridoxal phosphate (0·2 mM).

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TABLE 3. EFFECT OF NITROGEN SUPPLY ON THE PYRIDOXAL-5'-PHOSPHATE AND PYRIDOXAMINE-5'-PHOSPHATE CONTENT OF *Lemna minor*

"Nitrogen" in growth medium (mM)		(nmoles/g fr. wt)	
$Ca(NO_3)_2$	KNO_3	Pyridoxal phosphate	Pyridoxamine phosphate
0·5	0·5	0·15	0·61
2·5	2·5	0·20	1·00
5·0	5·0	0·08	2·08
10·0	10·0	0·08	3·42
25	25	0·07	2·43

Lemna grown on the medium used by Trewavas[26,27] but with the nitrogen content varied as indicated below. Pyridoxal phosphate and pyridoxamine phosphate determined after 3 weeks growth.

the same ingredients except that the nitrogen content was varied. The cultures were grown for 3 weeks and then free pyridoxal phosphate and pyridoxamine phosphate was determined as described in the Experimental (Table 3).

Haplopappus gracilis cultures were grown on the nutrient medium described by Murashige and Skoog.[28] Subcultures were made on to media containing different concentrations of nitrates. When the KNO_3 concentration of the medium was reduced an equal concentration of KCl was added. After growth for 3 weeks the cultures were assayed for pyridoxal phosphate and pyridoxamine phosphate. The results are shown in Table 4 for two strains of the culture.

TABLE 4. EFFECT OF NITROGEN SUPPLY ON THE PYRIDOXAL SUPPLY ON THE PYRIDOXAL-5'-PHOSPHATE AND PYRIDOXAMINE-5'-PHOSPHATE CONTENT OF *Happlopappus gracilis*

Strain	Nitrate concentration (mM)	(nmoles/g fr. wt) Pyridoxal phosphate	Pyridoxamine phosphate
YA	9·4	1·45	2·27
	18·8	1·52	2·66
	60·0	1·55	2·20
	101·2	1·00	2·84
	142·4	0·96	3·18
Y	9·4	1·64	1·72
	18·8	1·25	2·27
	60·0	1·48	1·88
	101·2	1·13	2·57
	142·4	0·98	3·12

Happlopappus grown on the medium used by Murashige and Skoog[28] but with the nitrogen content varied as indicated below. Pyridoxal phosphate and pyridoxamine phosphate determined after 3 weeks growth.

DISCUSSION

The results reported in this paper show that glutamate dehydrogenase from pea mitochondria is appreciably inhibited by low concentrations of pyridoxal phosphate. The time and concentration dependence of the inhibition and the reversibility of the inhibition in the presence of the enzymes substrates, are very similar to those reported for glutamate dehydrogenase from animal sources.[10,11] This similarity suggests that the mechanism of

[28] MURASHIGE, T. and SKOOG, F. (1962) *Physiol. Plant.* **25**, 473.

inhibition in the plant and animal enzymes is similar—involving the formation of a Schiff base between pyridoxal phosphate and a γ-amino group of a lysine residue.[29]

This interaction may simply be an interesting piece of chemistry in which a group specific agent attacks an isolated protein. On the other hand, the reversibility of the reaction in the presence of the substrates for reductive amination argues for the physiological significance of the reaction. We have measured the concentration of free pyridoxal phosphate in two plants and the concentrations (*c.* 0·1 μM in *Lemna minor* and *c.* 1·6 μM in *Haplopappus gracilis*) is comparable with the concentration reported in rat liver (3·3 μM).[30] If we assume that the vacuole occupies 90% of the cell volume and contains no pyridoxal phosphate, the concentration of pyridoxal phosphate in the cytoplasm lies between 2 and 16 μM. The plant glutamate dehydrogenase is clearly inhibited by these concentrations (Fig. 2) and if the pyridoxal phosphate is concentrated in the mitochondria, where glutamate dehydrogenase is located, it is likely that the enzyme will be significantly inhibited. The data also shows that the concentration of pyridoxal phosphate varies with the amount of nitrogen (nitrate) supplied to the plants and this again suggests possible physiological significance. However, before the suggestion can be taken too far it is necessary to establish that there is selective inhibition of enzymes by pyridoxal phosphate. We have examined 11 enzymes present in pea epicotyls. In addition to glutamate dehydrogenase, four of these enzymes show significant inhibition by pyridoxal phosphate and all four are involved in carbohydrate metabolism. These results must be regarded as preliminary, but they are consistent with ascribing physiological significance to inhibition by pyridoxal phosphate. The relationship between carbohydrate and nitrogen metabolism was investigated by Gregory and Sen in 1931[31] and their interpretation has become known as the protein cycle. Steward *et al.*[32–35] consider that as much as 50% of the respired CO_2 comes from protein turnover. Whilst this particular estimate may be unduly high,[27] few would deny the close inter-relationship between nitrogen and carbohydrate metabolism and there is an extensive literature showing the close relationship between respiration and the supply of nitrogenous nutrients.[36–38] The corelation between nitrogenous nutrition and the ratio pyridoxal phosphate/pyridoxamine phosphate together with the observed inhibition of glutamate dehydrogenase and some glycolytic enzymes by pyridoxal phosphate suggests a possible molecular basis for the relationship between nitrogen supply and respiration.

It is suggested that the nitrogen status of a plant is monitored by the ratio pyridoxal phosphate/pyridoxamine phosphate and that certain enzymes of glycolysis and glutamate dehydrogenase respond to this ratio or to the concentration of pyridoxal phosphate. Under conditions of nitrogen starvation the concentration of pyridoxal phosphate will be high and will inhibit glycolysis thereby restricting the supply of carbohydrates to the Krebs cycle. The simultaneous blocking of glutamate dehydrogenase prevents the loss of organic nitrogen by deamination of glutamate. When the nitrogen supply is increased the ratio

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pyridoxal phosphate/pyridoxamine phosphate falls, the inhibition of glycolysis will be removed, the supply of carbon skeletons for the synthesis of amino acids will increase and the simultaneous activation of glutamate dehydrogenase ensures that inorganic nitrogen is brought into organic combination.

The literature contains many observations consistent with these proposals. For example the addition of ammonia to *Chlorella* produces changes in the levels of metabolites consistent with an activation of glucose-6-phosphate dehydrogenase, glutamate dehydrogenase and pyruvate kinase[39]—all three enzymes are inhibited by pyridoxal phosphate. However the potential lack of specificity associated with the attack of pyridoxal phosphate or lysine residues does not inspire confidence in the proposals. Consequently the report[40] that *Lemna minor* contains a NADPH nitrate reductase which is inhibited by pyridoxamine phosphate is of special importance to the proposals.

EXPERIMENTAL

Plant material. Pea seeds (*Pisum sativum* var. Alaska) were soaked overnight in running tapwater then planted in moist vermiculite and germinated for 6 days in the dark at 25°. *Lemna minor* was grown under sterile conditions as described by Trewavas.[26,27] Cultures of *Happlopappus gracilis* supplied by Dr. N. Sunderland (John Innes Institute, Norwich, U.K.) were grown on a medium described by Murashige and Skoog.[28]

Chemicals. All general chemicals were of the highest purity available commercially. Fine chemicals were purchased from Sigma Chemical Co. London and Boehringer Corp. (London).

Preparation of glutamate dehydrogenase. All operations unless otherwise stated were carried out at 0–4°. Pea epicotyls were ground with sand in a mortar in TES buffer (pH 7·4, 50 mM) containing sucrose (0·5 M) to produce a 2/1 (W/V) homogenate. The extract was squeezed through nylon and centrifuged at 500 *g* for 10 min. The supernatant was centrifuged at 10000 *g* for 20 min, the mitochondrial precipitate was resuspended in the extraction medium and then recentrifuged. The mitochondrial pellet was resuspended in a small volume of TES buffer pH 7·4 50 mM then frozen and thawed before being centrifuged at 48000 *g* for 30 min to give a pale yellow supernatant which was adjusted to pH 6 with HOAC (1 M) and then heated in a water bath at 70° for 5 min. Denatured protein was removed by centrifuging at 15000 *g* for 10 min and the clear supernatant treated with 0·2 vol. calcium phosphate gel (8·5 mg dry weight/ml). The precipitate collected by centrifugation was washed twice with NaCl (0·5 M) and the glutamate dehydrogenase subsequently eluted with 12% $(NH_4)_2SO_4$ in potassium phosphate buffer (pH 8·0, 0·5 M). The supernatant was then passed through a column (1·5 × 40 cm) of Sephadex G-200 previously equilibrated with TES buffer (pH 7·4, 50 mM). 2 ml fractions were collected and the fractions containing max glutamate dehydrogenase activity were combined. The purification achieved is shown in Table 1.

Assay of glutamate dehydrogenase. Initial velocity measurements were made with a Unicam SP 500 spectrophotometer coupled to a Vitatron strip chart recorder and fitted with a constant temperature compartment maintained at 30°. Oxidative deamination was measured as the increase in E_{340} when the assay mixture contained L-glutamate (13·3 mM), NAD^+ (0·2 mM) enzyme, TES buffer (pH 7·5, 50 mM) in a final volume of 3 ml. Reductive amination was measured as the decrease in E_{340} when the assay mixture contained 2-oxoglutarate (2 mM), NADH (0·2 mM) NH_4Cl (0·13 M) enzyme and TES buffer (pH 7·5, 50 mM) in a final vol. of 3 ml.

Preparation of enzyme extract from peas. Pea seedlings were harvested and the epicotyls (200 g) ground with 400 ml of K phosphate buffer (50 mM, pH 7·4), the juice was squeezed through nylon cloth and centrifuged at 20000 *g* for 15 min. The supernatant was brought to 60% saturation with $(NH_4)_2SO_4$ and the precipitate collected by centrifuging at 10000 *g* for 15 min. The precipitate was dissolved in 50 ml K phosphate buffer (50 mM pH 7·4) and then centrifuged at 15000 *g* for 15 min. The supernatant was dialysed overnight against 3 l. of 20 mM K phosphate buffer (pH 7·4) and then centrifuged at 15000 *g* for 10 min before being used for enzyme assays.

Samples of the crude extract (10 ml) were incubated with 40 ml K phosphate buffer (pH 7·5, 50 mM) in the presence and absence of pyridoxal phosphate for 30 min at 30°. An aliquot (1 ml) corresponding to 0·2 ml of crude extract was used for the enzyme assays except when NADH or NADPH was a component of the assay system—in these conditions 0·4 ml of the incubated extract was used.

Enzyme assays. Malic enzyme, alcohol, lactate, malate, isocitrate, glucose-6-phosphate and 6-phosphogluconate dehydrogenases were measured as described.[41] Shikimate dehydrogenase by the method of Balinsky and

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Davies,[42] phosphoenol-pyruvate carboxylase by the method of Wong and Davies[43] and phenylalanine ammonia lyase by reading the increase in E_{290} in the presence of L-phenylalanine (20 mM).[44]

Determination of pyridoxal-5'-phosphate and pyridoxamine-5'-phosphate. Samples of tissue (3 g) were frozen with liquid nitrogen and homogenized in a mortar with pure quartz sand and 5 ml of K phosphate buffer (pH 7·4, 50 mM). The supernatant obtained after centrifuging this extract for 10 min at 20000 *g* was rapidly filtered in an Amicon ultrafiltration cell (model 10-PA) equipped with a Diaflo ultra filter (UM 10) which retained substances of molecular weight higher than 10000. This filtration is used to remove protein in such a way that the enzyme bound pyridoxal phosphate and pyridoxamine phosphate do not dissociate from the proteins. Pyridoxal phosphate was determined in aliquots of the filtrate by the method of Adams.[45] Pyridoxamine phosphate was converted into pyridoxal phosphate by transamination with glyoxylic acid as described by Toepfer *et al.*[46]

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