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THE SYNTHESIS OF GLUTAMATE AND THE CONTROL OF GLUTAMATE DEHYDROGENASE IN PEA MITOCHONDRIA

DAVID D. DAVIES and ARTUR N. TEIXEIRA*

School of Biological Sciences, University of East Anglia, Norwich, Norfolk, England

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Abstract—The synthesis of glutamate from α-oxoglutarate and NH_4^+ by pea seedling mitochondria has been demonstrated under certain defined but non-physiological conditions. Malate acts as a hydrogen donor for the synthesis of glutamate but isocitrate is more effective, whilst succinate, in the presence or absence of ATP, is a poor donor of hydrogen. Glutamate dehydrogenase has been purified from pea mitochondria and from the cytosol. The similarities between the two preparations are interpreted to mean that the soluble glutamate dehydrogenase is released from the mitochondria during isolation. The kinetics of the mitochondrial enzyme and the effect of various metabolites on its activity have been examined. The results are discussed in relation to the proposed role of this enzyme and it is suggested that the ratio NADH–NAD^+ may play a role in the control of glutamate metabolism.

INTRODUCTION

Glutamate dehydrogenase is the main portal in plants for the entry of ammonia into organic combination [1]. Accepting the general proposition that the reducing potential of NADH is utilized for the production of ATP whilst NADPH provides the reducing potential for biosynthesis [2], it appears likely that NADPH is involved in the reductive amination forming glutamate. However, glutamate dehydrogenases from animal sources can utilize NADH or NADPH almost equally well and the enzyme is subject to complex regulation. In micro-organisms possessing separate NAD and NADP specific glutamate dehydrogenases, the NADP specific enzyme is considered to have a biosynthetic function [3]. Fungi of the Phycomycetes possess only a NAD specific enzyme which has complex regulatory properties permitting it to serve both catabolic and anabolic functions [4]. The control of glutamate dehydrogenase has been admirably reviewed by Goldin and Frieden [5], unfortunately without reference to plants.

The presence of NADP specific glutamate dehydrogenase in chloroplasts has been reported [6] and this enzyme presumably plays a role in relation to photosynthesis. The non-green parts of plants possess glutamate dehydrogenase which is active with NAD and NADP. Some workers [7, 8] suggest the existence of separate NAD and NADP specific enzymes, whilst others [9–11] propose a single enzyme lacking specificity for the coenzyme.

The isoenzymes of NAD-glutamate dehydrogenase are well documented [12–16]. There is evidence that the pea isoenzymes are conformers [15] and that one of the isoenzymes increases when a tissue is supplied with ammonia [14, 17]. The distribution of the isoenzymes within the cell is not clear. In mung beans [9], pea epicotyls [18], soybean cotyledons [19], corn leaves [20] and castor beans [7] the enzyme is present in mitochondria and the soluble activity is considered to be solubilized mitochondrial enzyme. However, in pumpkins [13] most activity is found in the soluble fraction and the soluble enzyme differs from the particulate enzyme in being more sensitive to NH_4^+ inhibition and in being activated by metal ions after $(NH_4)_2SO_4$ fractionation; Yakoleva *et al.* [21] on

* Present address: Estacao Agronomica Nacional, Oeiras, Portugal.

Table 1. Purification of mitochondrial glutamate dehydrogenase from pea epicotyls

Fraction	Volume (ml)	Units	Specific activity	Yield (%)	Purification
Crude extract	15	0·317	0·044	100	—
After centrifuging at 48000 *g*	13	0·296	0·084	81	1·8
After G-25	17	0·214	0·091	76	2
After heating at 70°	16	0·205	0·27	68	6
After calcium phosphate gel	10	0·190	1·34	40	30
After Sephadex G-200:					
Combined Fractions 25–28	8	0·193	6·65	32	150

the basis of differential adsorption on calcium phosphate gel conclude that the mitochondrial and supernatant enzymes are different proteins.

Against this complex background a number of attempts have been made to study the regulatory properties of glutamate dehydrogenase [11, 15, 19, 22]. These studies have established that glutamate dehydrogenase has a number of effectors but it has not been possible to present a scheme which is physiologically significant.

This paper examines the capacity of pea epicotyl mitochondria to synthesize glutamate and reports the properties of the glutamate dehydrogenase of pea epicotyls in relation to the possible regulation of the enzyme by the ratio NAD–NADH.

RESULTS

Preparation of glutamate dehydrogenase

The preparation of the mitochondrial and "soluble" forms of glutamate dehydrogenase from pea epicotyls is outlined in Tables 1 and 2 respectively.

Synthesis of glutamate by mitochondria

The synthesis of glutamate from α-oxoglutarate, NH_3 and various hydrogen donors has been observed with animal mitochondria [23, 24]. Using similar, non-physiological conditions, we have studied glutamate synthesis by pea mitochondria. Washed mitochondria were incubated with the standard reaction mixture supplemented with [5^{14}-C-2]-oxoglutarate, organic acids and ATP as indicated in Table 3. After incubation under anaerobic conditions for 20 min at 30°, the incorporation of radioactivity into amino acids was determined and the increase in glutamate and aspartate measured. Negligible changes in both glutamine and asparagine were observed. These results suggest that isocitrate is an efficient donor of hydrogen for the synthesis of glutamate resembling those of Tager and Slater [23] for rat-liver mitochondria, but not those of Klingenberg *et al.* [24] in which isocitrate was found to be a poor supplier of hydrogen. Accordingly, we carried out the same procedure as described above with rat-liver mitochondria. The results are shown in Tables 4 and 5.

Utilization of tritiated substrates as hydrogen donors for glutamate synthesis

In principle, the efficiency of malate, isocitrate and succinate as hydrogen donors for glutamate synthesis can be determined by measuring the incorporation of tritium into glutamate from appropriately labelled substrates. In practice the experiment is made difficult by an exchange reaction between water and the hydrogen on the α carbon of glutamate catalysed by glutamate–aspartate aminotransferase. We have attempted to overcome

Table 2. Purification of "soluble" glutamate dehydrogenase from pea epicotyls

Fraction	Volume (ml)	Units	Specific activity	Yield (%)	Purification
Crude extract	100	0·015	0·0009	100	—
After heating at 70°	100	0·011	0·0017	75	2
After $(NH_4)_2SO_4$	12	0·0425	0·0017	25·5	14
After calcium phosphate gel	8	0·039	0·017	20	19
After Sephadex G-200:					
Combined Fractions 22–25	8	0·031	0·062	16·5	72

Table 3. Comparison of hydrogen donors for the synthesis of glutamate and aspartate by pea mitochondria

Addition	Incorporation of ^{14}C into amino acids (cpm × 10^{-2})	Recovery of ^{14}C in amino acids (%)	Δ Glutamate (nmol)	Δ Aspartate (nmol)
Experiment (a)				
None	226	4·0	50	40
ATP	293	5·2	81	76
Malate	369	6·5	125	200
Malate + ATP	302	5·4	80	390
Isocitrate	751	13·3	457	80
Isocitrate + ATP	540	9·6	395	75
Succinate	244	4·2	92	45
Succinate + ATP	212	3·9	80	30
Experiment (b)				
None	655	11·4	217	410
ATP	761	13·4	310	825
Malate	950	16·7	619	985
Malate + ATP	802	14·0	540	1690
Isocitrate	1230	21·5	1489	570
Isocitrate + ATP	1150	20·2	1219	890
Succinate	742	13·2	350	210
Succinate + ATP	694	12·1	285	100

The standard reaction mixture (2 ml) was supplemented with NH_4Cl (10 mM) 2-oxoglutarate-5-[^{14}C] (2·5 mM, 0·5 μCi) together with ATP (5 mM). L-malate (5 mM) or succinate (5 mM) or DL-isocitrate (10 mM) as indicated. In experiment (a) the mitochondrial suspension (1 ml) added to each tube contained 10·8 mg protein. In experiment (b) the mitochondrial suspension contained 32·5 mg protein. Incubation in nitrogen, temp 30°, time 20 min.

this difficulty by using isonicotinic acid hydrazide [25] and D-cycloserine [26] to block this exchange reaction. These compounds, in concentration up to 40 mM, had no inhibitory effect on the activities of malate, isocitrate or glutamate dehydrogenase, but cycloserine (10 mM) and isonicotinic acid hydrazide (15 mM) inhibited the aspartate aminotransferase reaction by 94 and 90% respectively. Unfortunately, the per cent incorporation from tritiated substrates into glutamate was low, though differences in the efficiency of hydrogen donors could always be detected and isocitrate was always significantly the most effective donor (Table 6).

Comparison of mitochondrial and soluble glutamate dehydrogenase

Comparison of the purified mitochondrial and supernatant enzyme preparation (Tables 1 and 2) suggests that they are the same. The evidence may be summarized as follows: (a) the ratio V_{max} NADH oxidation/V_{max} NAD^+ reduction was 25 for both preparations; (b) both preparations have the same pH optima in both directions of assay; (c)

Table 4. Comparison of hydrogen donors for the synthesis of glutamate and aspartate by rat liver mitochondria

Addition	Incorporation of ^{14}C into amino acids (cpm × 10^{-2})	Recovery of ^{14}C in amino acids (%)	Δ Glutamate (nmol)	Δ Aspartate (nmol)
None	303	5·4	150	50
ATP	492	8·8	433	36
Malate	802	14·3	805	120
Malate + ATP	1501	26·8	1110	1630
Isocitrate	1727	29·1	2293	50
Isocitrate + ATP	1934	34·4	2650	80
Succinate	523	10·8	483	36
Succinate + ATP	1822	32·5	1782	680

Conditions as specified in Table 3 except pea mitochondria replaced by rat liver mitochondria (9·4 mg protein/ml).

Table 5. Comparison of hydrogen donors for the synthesis of glutamate and aspartate by rat liver mitochondria

Additions	Δ Glutamate (nmol)	Δ Aspartate (nmol)
None	183	9
ATP	299	12
Malate	648	280
Malate + ATP	465	1160
Isocitrate	3091	25
Isocitrate + ATP	2861	48
Succinate	316	230
Succinate + ATP	1280	690

Conditions as specified in Table 4 except DL-*iso*citrate (10 mM) replaced by threo D(+) *iso*citrate (5 mM) and the protein concentration of the mitochondrial suspension was 15·7 mg/ml.

Table 6. Comparison of hydrogen donors for glutamate synthesis in pea mitochondria

Additions	Recovery of radioactivity in glutamate (%)
L-Malate (5 mM) + DL(2-^{3}H) malate 5 μCi	0·64
DL-Isocitrate (10 mM) + DL(2-^{3}H) isocitrate 10 μCi	1·07
Succinate (5 mM) + (2·3-^{3}H) succinate 2·5 μCi	0·03
Succinate (5 mM) + (2·3-^{3}H) succinate 2·5 μCi + ATP (5 mM)	0·04

The standard reaction mixture (2 ml) was supplemented with NH_4Cl (20 mM), 2-oxoglutarate (5 mM), cycloserine (20 mM) and tritiated organic acids as indicated. Mitochondria (1 ml containing 8·3 mg protein) added to each tube. Incubation in N_2, temperature 30°, time 20 min). Results are expressed as per cent recovery corresponding to the enzymatically active isomer.

Michaelis constants for substrate and coenzymes are virtually the same (see Table 7); (d) the enzyme activity of both preparations is inhibited to virtually the same extent by citrate, fumarate, succinate ADP, ATP and EDTA; (e) both preparations show high specificity for 2-oxoglutarate (amination system) and L-glutamate (deamination system), and (f) both enzymes have the same molecular weight.

The soluble and mitochondrial enzymes adsorb on calcium phosphate gels to different degrees during purification. The mitochondrial enzyme adsorbs tightly whereas the soluble enzyme remains in the supernatant after treatment with calcium phosphate. Since the protein and salt concentrations in the two preparations are widely dissimilar, this different behaviour is not surprising. We suggest, therefore, that the soluble glutamate dehydrogenase of pea epicotyls is released from the mitochondria during isolation. Accordingly, we present detailed information only for the purified mitochondrial enzyme.

Molecular weight. Determinations of the approximate MW were carried out by chromatography on Sephadex G-200 [27]. Yeast alcohol dehydrogenase, catalase and cytochrome *c* were used as markers. The MW of the mitochondrial enzyme was found to be 230000.

pH Optima. Under the standard conditions of assay, the optimum for the aminating reaction was found to be 8·2 and for the deaminating reaction 8·2–8·4. Enzyme activity was found to vary with the buffer. Putting the deaminating activity in 100 mM Tris (hydroxymethyl)methyl-2-aminoethanesulphonic acid (TES) at 100%, the activity with 100 mM Tris was 65% and with 100 mM sodium pyrophosphate 50%. Putting the aminating activity in 100 mM TES at 100% the activity with Tris was 75% and with pyrophosphate 60%.

Coenzyme specificity. The rate of amination with NADH as the coenzyme was about 20-fold that

Table 7. Michaelis constants for glutamate dehydrogenase from different sources K_m (μM)

Substrate	Beef liver [28]	*Neurospora* NAD specific [29]	*Nitrosomas* NADP specific [30]	*Thiobacillus* NADP specific [31]	*Thiobacillus* NAD specific [31]	Soy bean [19]	Pea roots [11]	Pea epicotyls Mito-chondrial	Pea epicotyls Soluble
2-Oxoglutarate	700	4600	4300	7400	667	1200	3300	620	590
NADH	115	550	—		4	15	860	26	31
NADPH	35	—	49	77	—	—	—	660	650
NH_4^+	3200	17000	16000	7500	500	9400	38000	70000	56000
Glutamate	1800	5500	6700	35500	13300	8000	7300	3500	6200
NAD^+	70	330	—	—	190	210	650	590	280
$NADP^+$	95	—	7·9	61	—	—	—	—	—

— Means not determined.

Table 8. Michaelis constants for pea mitochondrial glutamate dehydrogenase at various pH values K_m (μM) at stated pH

Substrate	6·5	7·4	7·6	8·2	9·0
NAD	—	130	—	590	K_{m_1} 360 K_{m_2} 29
Glutamate	—	4900	—	3500	11000
NH_4^+	22000	—	29000	70000	35000
2-Oxoglutarate	70	—	200	620	270
NADH	12·5	—	18	26	31

obtained with NADPH, for both mitochondrial and soluble enzymes.

Michaelis constants. When assayed at pH 8·2 the enzyme showed normal Michaelis–Menten kinetics with the exception that concentrations of NH_4^+ in excess of 0·15 M produced inhibition. The kinetic constants for the mitochondrial and soluble enzymes are presented in Table 7 together with constants reported for a number of preparations from other sources. The effect of pH on the value of the Michaelis constants was also determined and the results are shown in Table 8. The two values given for K_m (NAD) at pH 9·0 reflect the biphasic response of the enzyme (Fig. 1). 2-Oxoglutarate also showed non-Michaelis–Menten kinetics, being concave downwards (Fig. 2). The K_m values given in Table 8 are for the linear parts of the curves.

Inhibition of glutamate dehydrogenase by p-chloromercuribenzoate and EDTA

The enzyme was found to be inhibited by the thiol reagent *p*-chloromercuribenzoate in both directions of enzyme assay, but more extensively in the deamination reaction (53% inhibition at 100 mM). Activity was completely restored by the addition of reduced glutathione at concentrations 2–3 times the concentration of *p*-chloromercuribenzoate. Assayed under standard conditions, the deamination reaction was inhibited 60% by 0·1 mM EDTA, and under standard conditions except that the pH was 8·2, the amination reaction was inhibited 78% by 0·1 mM EDTA. The inhibition was relieved by a number of inorganic cations including Mn, Co, Zn, Ca.

Metabolites. ATP behaved as a competitive inhibitor; with respect to NADH in the amination reaction and with respect to NAD in the deamination reaction. ADP also behaved as a competitive inhibitor but AMP produced only slight inhibition. The inhibition produced by a number of other metabolites is shown in Table 9.

Product inhibition. 2-Oxoglutarate, NH_4^+ and glutamate behaved as competitive inhibitors (K_i 1·8, 0·11 at pH 8·0 and 12 at pH 7·5 respectively).

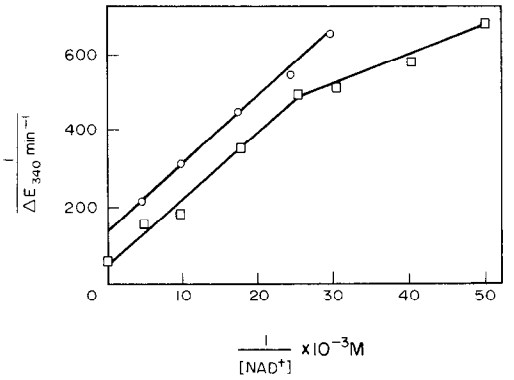

Fig. 1. Double reciprocal plot of rate against NAD concentration at two pH values. Assay system: glutamate (13·3 mM); enzyme (0·14 mg protein) buffer; and NAD^+ varied as indicated in a total volume of 3 ml. □——□ Tris buffer pH 9·0 (0·1 M); ○——○ Potassium phosphate buffer pH 7·4 (0·1 M).

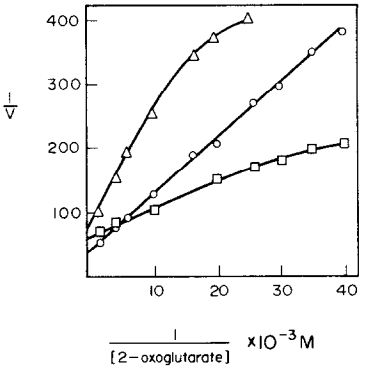

Fig. 2. Double-reciprocal plot of rate against 2-oxoglutarate concentration at different pH values. Assay system: NADH (0·2 mM); NH_4Cl (0·13 M); enzyme (0·06 mg protein); 2-oxoglutarate; and buffer as indicated in a final vol. of 3 ml. △——△ Tris buffer pH 9·0, 0·1 M; ○——○ Potassium phosphate buffer pH 7·6, 0·1 M; □——□ Potassium phosphate buffer pH 6·5, 0·1 M.

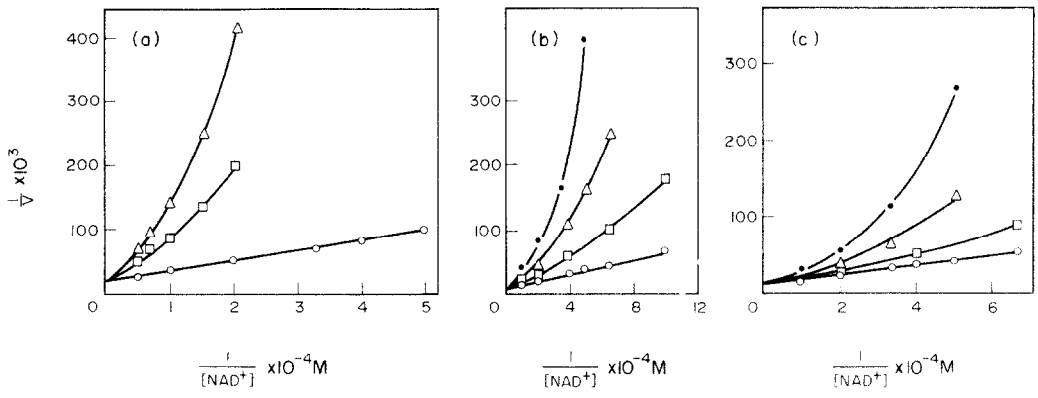

Fig. 3. Effect of NADH on the activity of glutamate dehydrogenase measured in the direction of deamination Standard conditions of assay except NAD^+ and buffer varied as indicated. A—HEPES buffer (pH 7·0, 0·1 M). B—HEPES buffer (pH 8·0, 0·1 M). C—Tris buffer (pH 9·0, 0·1 M). Concentration of NADH, ○——○; control—□——□ 2·5 μM, △——△ 5 μM, ●——● 7·5 μM; enzyme 0·25 mg protein.

NAD^+ showed anomalous kinetics at relatively high concentrations, but these effects have not been examined in detail. NADH was found to be a strong inhibitor of the deamination reaction. In the form of double reciprocal plots, the curves were concave upwards (Fig. 3). Plotted by the method of Johnson *et al.* [32] (log $V_0/V_I - 1$ vs log[I]) a linear relationship was observed indicating Hill coefficients greater than unity ($n = 2·3$ when $[NAD^+] = 20\,\mu M$ and $n = 1·4$ when $[NAD^+] = 50$ mM). When the data are plotted in the form of a Dixon plot ($1/V$ vs [I]) the relation

Table 9. Effect of metabolites on the activity of glutamate dehydrogenase. Activity measured under standard conditions described in text

Addition	Concentration (mM)	% Activity amination	% Activity deamination
Citrate	4·0	76	80
Isocitrate (DL)	6·7	100	—
	10	—	90
Fumarate	6·7	73	—
	10	—	80
Succinate	6·7	82	—
	10	—	100
Fructose diphosphate	6·7	100	—
	10	—	100
Aspartate	10	100	—
Asparagine	10	100	—
Glutamine	10	100	—
Alanine	10	100	—
AMP	0·5	—	100
	3·3	95	—
	5·0	—	80
ADP	0·5	—	67
	3·3	92	—
	5·0	—	42
	6·7	80	—
ATP	0·5	—	50
	3·3	85	—
	5·0	—	25
	6·7	71	—

— Not determined.

is curved but approximates to linearity when $1/v$ is plotted vs the square of the inhibitor concentration $[I]^2$.

DISCUSSION

The capacity of plant mitochondria to *oxidize* glutamate has been demonstrated by a number of workers [33–35]. However, this paper is concerned with the possibility that mitochondria from pea epicotyls can synthesize glutamate. By following earlier methods [23, 24] we have demonstrated that under certain defined conditions, pea mitochondria can synthesize glutamate from 2-oxoglutarate and NH_3, utilizing the reducing potential provided by isocitrate or malate. The conditions under which synthesis has been demonstrated are unphysiological and the results should be treated with caution when extrapolated to *in vivo* situations. Nevertheless a number of points deserve comment.

Glutamate synthesis in pea mitochondria does not appear to require ATP—rather ATP inhibits glutamate synthesis. This contrasts with the ATP requirement for glutamate synthesis by rat liver mitochondria reported previously [23, 24] and confirmed by the results presented in Table 4. Succinate which is a source of reducing potential for glutamate synthesis in rat liver mitochondria appears to be a poor source of hydrogen for glutamate synthesis in pea mitochondria. Reversed electron transport from succinate to NAD^+ and driven by ATP has been demonstrated in mung bean mitochondria [36–38] and could be responsible for the limited synthesis of glutamate by pea mitochondria when succinate is the hydrogen source, but it should be noted that ATP inhibits the synthesis of glutamate. ATP stimulates the synthesis of aspartate with malate or isocitrate as the hydrogen donor but ATP inhibits the synthesis of aspartate when succinate is the hydrogen donor.

Isocitrate appears to be an effective hydrogen donor for glutamate synthesis in pea mitochondria [cf. 23, 24]. The experiments with tritiated isocitrate establish a transfer of hydrogen from isocitrate to glutamate but do not provide information on whether NADH or NADPH is the transferring molecule.

In rat liver mitochondria evidence has been presented that NADPH is preferentially used for glutamate synthesis [39, 40]. However, glutamate dehydrogenase isolated from pea mitochondria is much more active with NADH than with NADPH. We have considered the possibility that there is a special link between NAD specific isocitrate dehydrogenase and glutamate dehydrogenase in plants—isocitrate providing the carbon skeleton and the reducing potential for glutamate synthesis. We have shown [41] that NAD specific isocitric dehydrogenase from peas is "A" specific for the nicotinamide ring whilst glutamate dehydrogenase is "B" specific. This situation fits Velick's [42] correlation between opposite stereospecificities and physiological function. Whether or not plants possess an organized unit relating isocitrate oxidation and glutamate synthesis, it is necessary to assume some form of control relating the passage of the carbon skeleton of isocitrate around the Krebs cycle to its incorporation into glutamate. Similarly the transfer of hydrogen from NADH to glutamate must be controlled relative to the transfer to oxygen with its associated production of ATP. With these considerations in mind we have examined the properties of the glutamate dehydrogenase present in mitochondria.

The reaction catalysed by glutamate dehydrogenase is freely reversible but a consideration of the kinetic constants might indicate the direction in which the enzyme is "designed" to operate. Cleland [43] argues that to maximize the rate of reaction in a particular direction, the Michaelis constants for the products should be smaller than those for the substrates. This argument follows from the general Haldane relationship in equation 1

$$K_{eq} = V_1/V_2 \times K_P/K_s \qquad (1)$$

where K_{eq} is the equilibrium constant for the overall reaction, K_p and K_s are Michaelis constants for the product and substrate, v_1 is the maximum velocity s to p and V_2 is the maximum velocity p to s. Cleland places importance on maximizing the ratio V_1/V_2. However, as Atkinson [44] has pointed out, a high value of V_1 is not incompatible with a high value of V_2 and on metabolic and evolutionary grounds he argues that K_s should be smaller than K_p. In the case of pea glutamate dehydrogenase K_{NADH} is an order of magnitude lower than K_{NAD}, whilst $K_{oxoglutarate}$ is close to $K_{glutamate}$. This situation would suggest that the pea

mitochondria glutamate dehydrogenase is "designed" to catalyse the amination reaction. The affinity of the pea epicotyl glutamate dehydrogenase for NADH is 30 times that of the root enzyme (Table 7). Following Atkinson, this suggests that the root enzyme is less well "fitted" for glutamate biosynthesis. However, using the same argument, the data for the NAD specific glutamate dehydrogenase of *Thiobacillus novellus* [31] suggests that it is even better "designed" for the amination reaction, whereas Lé John and McCrea [45] suggest that the enzyme acts in a purely catabolic manner in the cell. It therefore appears that we cannot use the data from the intact mitochondrial studies or the kinetic data for glutamate dehydrogenase to establish the physiological direction in which the enzyme functions; it may well be that this is under metabolic control.

The kinetic studies of the enzyme show that its properties are in general very similar to those reported for the NAD-glutamate dehydrogenase from other plants and organs. Since allosteric properties may be lost during purification we have compared the properties of crude and purified enzymes—substantially the same results were to be obtained. The data on inhibition by EDTA, ATP, organic and amino acids is similar to that reported by King and Wu [19] and Pahlich and Joy [11]. However, we wish to draw attention to the marked inhibition of the deamination reaction by low concentrations of NADH. The inhibition shows the special kinetic characteristics which we have come to expect of control enzymes. NADH would be expected to inhibit the deamination reaction simply by mass action and competitive inhibition. The data obtained (Fig. 3 and described in text) shows the co-operative nature of the inhibition but it does not seem profitable at this stage to discuss the inhibition in terms of the general allosteric models which have been reported.

At the physiological level, control by the ratio NAD–NADH seems plausible. Thus, if the level of the Krebs cycle acids in the mitochondria is high and the ratio NAD–NADH correspondingly low, the deamination of glutamate will be inhibited. Should the level of Krebs cycle acids fall and the ratio NAD–NADH rise the inhibition of the deamination reaction would disappear and the Krebs cycle could be replenished at the expense of glutamate. However, when the Krebs cycle acids are being replenished by CO_2 fixation, it seems likely that the deamination reaction would be inhibited. It is interesting to note that phosphoenolpyruvate carboxylase in inhibited by glutamate [46]. Thus control of carboxylation coupled with control of glutamate dehydrogenase should ensure that the Krebs cycle can simultaneously exert its respiratory and synthetic roles.

Finally, we must mention the worrying fact that the enzyme has such a poor affinity for ammonium ions. Most glutamate dehydrogenases have been found to have low affinities for ammonium and Meister [47] suggested that ammonia rather than ammonium may be the enzymatically active form. Fisher and McGregor [48] on the basis of inhibition studies concluded that ammonium rather than ammonia is the substrate for the reaction. The speed with which ammonia reacts with water makes it difficult to examine Meister's proposal, but the association of glutamate dehydrogenase with mitochondria may provide a lipid rich "microclimate" in which ammonia is more readily available to the dehydrogenase. A careful examination of higher plants for an alternative route for glutamate synthesis should be undertaken and in particular the possibility that glutamine provides the nitrogen for the reductive synthesis of glutamate from α-oxoglutarate in a manner similar to the glutamate synthetase reaction found in bacteria [49]. Since completing this work several workers have demonstrated enzymes in plants similar to the bacterial glutamate synthetase.

EXPERIMENTAL

Buffers. All buffers were prepared with glass distilled H_2O. Tris under the trade name Trizma. *N*-tris (hydroxymethyl) methyl-2-aminoethane-sulphonic acid (TES) and 2-(*N*-2-Hydroxyethylpiperazin-*N*′-yl)ethane-sulphonic acid (HEPES) were obtained from Sigma, London.

Labelled compounds. Na [5-^{14}C]-2-oxoglutarate (17·5 mCi/m mole) and [2,3-^{3}H]-succinic acid (100 mCi/m mole) were obtained from the Radiochemical Centre, Amersham, Bucks, U.K. DL-[2-^{3}H] malic acid (19·6 mCi/m mol) and sodium DL-[2-^{3}H] isocitrate (8·1 Ci/m mole) were purchased from New England Nuclear Corp., U.S.A.

Enzymes. Yeast alcohol dehydrogenase, glutaminase and ox-liver catalase were obtained from Sigma, malate dehydrogenase from pig heart, glutamate dehydrogenase (suspended in glycerol) from ox-liver and aspartate aminotransferase from pig-heart were obtained from Boehringer Corp., London.

Other chemicals. NAD^+, NADH, NADPH, $NADP^+$ and oxaloacetic acid were obtained from Beohringer. L-glutamate (monosodium salt) AMP, ADP, ATP, *p*-chloromercuribenzoate, L-malate (monosodium salt), threo D$^{(-)}$ and DL-*iso*citrate

(trisodium salt), D-cycloserine, isonicotinic hydrazide and Dowex-50 W resin (100–200 mesh 8% cross linkage) were obtained from Sigma. Sephadex G-25 and G-200 were obtained from Pharmacia, Sweden. Most other chemicals were obtained from Fisons Scientific Apparatus Ltd., U.K.

Protein measurement. Protein was determined by the spectrophotometric method of Warburg and Christian [50] and by the biuret method of Cleland and Slater [51].

Enzyme assays. The standard assay for deamination was carried out at pH 8·2 by measuring the increase in E_{340} associated with NAD^+ reduction. The assay mixture contained L-glutamate (13·3 mM), NAD^+ (0·2 mM), enzyme and TES buffer (50 mM, pH 8·2) in a vol. of 3 ml in silica cells of 1 cm light path. The initial velocity measurements were made at 30°. In some cases when the rate of reduction was low, the rates were measured using a fluorescence spectrophotometer coupled to a recorder. The wavelengths used were: exciter at 340 nm, analyser at 465 nm.

Amination was measured in 1 cm cuvettes as the decrease in E_{340}. The assay system contained 2-oxoglutarate (2 mM), NADH (0·2 mM) NH_4Cl (0·13 M) enzyme and TES buffer (50 mM, pH 8·0) in a final vol. of 3 ml.

Unit of enzyme activity. A unit of enzyme activity is defined as the oxidation or reduction of 1 μmol of coenzyme/min. Specific activity is defined as the units per mg of protein.

Preparation of mitochondria. Seeds of *Pisum sativa* (var Alaska) were soaked overnight in H_2O and germinated in the dark for 6 days at 25°. Etiolated pea epicotyls were harvested, chilled at 4° and ground in a mortar with sand in TES buffer (50 mM pH 7·4) with sucrose (0·5 M) (2 ml/g tissue). The macerated tissue was squeezed through nylon gauze and centrifuged for 10 min at 500 *g*. The supernatant was centrifuged at 10000 *g* for 20 min; the supernatant was used to prepare the "soluble" glutamate dehydrogenase and the mitochondrial pellet was resuspended in the extraction medium and again centrifuged for 10000 *g* for 20 min.

Preparation of mitochondrial glutamate dehydrogenase. Mitochondrial pellets were resuspended in a small volume of TES buffer (50 mM, pH 7·4) then frozen and thawed before being centrifuged at 48000 *g* for 30 min to give a pale yellow supernatant. The supernatant was adjusted to pH 6·0 with HOAc (1 M) and heated at 70° for 5 min. Denatured protein was removed by centrifuging at 10000 *g* for 5 min and the clear supernatant treated with 0·2 vol. of $CaPO_4$ gel (8·5 mg dry wt/ml). After centrifuging at 5000 *g* for 5 min, the ppt was washed 2× with NaCl (0·5 M) and the glutamate dehydrogenase subsequently eluted with $(NH_4)_2SO_4$ (12% w/v) in K phosphate buffer (0·5 M, pH 8·0). The supernatant was then passed through a column (2·5 × 40 cm) of Sephadex G-200 which had been equilibrated with TES buffer (50 mM pH 7·4). The eluate was collected in 2 ml fractions. The purification is shown in Table 1.

Purification of "soluble" glutamate dehydrogenase. The supernatant remaining after removing mitochondria was adjusted to pH 6·0 with HoAc (1 M) and heated at 70° for 5 min and then centrifuged at 10000 *g* for 10 min. The precipitate was discarded and $(NH_4)_2SO_4$ (420 g/l) added to the supernatant. After stirring for 15 min the preparation was centrifuged at 10000 *g* for 10 min. The ppt was redissolved in a small vol. of K phosphate buffer (20 mM pH 7·4) and again centrifuged to clear. The pH of the supernatant was adjusted to 6 and 0·2 vol. of Ca phosphate gel (8·5 mg dry wt/ml) added. The gel was removed by centrifuging at 5000 *g* for 5 min and the supernatant passed through a column (2·5 × 40 cm) of Sephadex G-200 previously equilibrated with TES buffer (50 mM pH 7·4). The eluate was collected in 2 ml fractions. The purification is shown in Table 2.

Determination of products. L-Glutamate was determined with glutamate dehydrogenase [52]; L-aspartate and L-asparagine were determined with malate dehydrogenase and aspartate-aminotransferase [53]; for the determination of asparagine, asparaginase was used in place of acid hydrolysis. L-Glutamine was determined with glutaminase and glutamate dehydrogenase [54].

Preparation of Dowex columns. Dowex-50 W was freed from heavy and fine particles and washed in hot HCl [55]. Glass columns (1 × 15 cm) fitted with sintered glass discs were filled to a height of 5 cm with resin.

Preparation of samples for analysis. The amino acid fractions eluted from the Dowex columns were freeze-dried which removes ammonium ions which would otherwise interfere with the determination of glutamate [52]. The residue from freeze drying was dissolved in 5 ml 6 N HCl. Aliquots (0·2–0·5 ml) of this sample were used for the determination of reaction products and for scintillation counting.

Counting of labelled compounds. The scintillation fluid contained PPO (7 g) and POPOP (600 mg) in 400 ml of 2-ethoxyethanol + 600 ml of toluene. Samples (0·2 ml or less) of radioactive solns were mixed with 15 ml of scintillation fluid for counting.

Standard conditions used for the synthesis of glutamate. The standard reaction mixture placed in Thunberg tubes contained EDTA (1 mM), Na arsenite (1 mM), sucrose (0·25 M), TES buffer (50 mM, pH 7·4) and the additions listed in the tables in a final vol. of 2 ml. The pH was adjusted to 7·4 and the mitochondrial suspension (1 ml) was placed in the arm of the Thunberg tube which was evacuated then filled with oxygen-free N_2, this operation was repeated 5×. The contents were mixed and after incubation at 30° for 20 min the reaction was stopped by the addition of 1 ml 60% $HCLO_4$.

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