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PARTIAL PURIFICATION AND KINETIC PROPERTIES OF GLUTAMIC DEHYDROGENASE FROM SOYBEAN COTYLEDONS

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Abstract—A NAD-dependent glutamate dehydrogenase (E.C. 1.4.1.2.) was isolated from soybean cotyledons (*Glycine max* L. var. Canadian No. 1) and purified 250-fold. The enzyme had maximum activity at pH 9·3 in the forward direction and at pH 8 in the reverse direction. The order of addition to the enzyme of the substrates NADH, 2-oxoglutarate and ammonia was the same as that for similar enzymes from micro-organisms. Metal ion inhibitor studies suggested that the enzyme was a metalloenzyme. ATP at concentrations of 1 mM and above was an inhibitor of enzyme activity in both directions. Kinetic data suggested that the enzyme was a metalloenzyme. ATP at concentrations of 1 mM and above was an inhibitor of enzyme activity in both directions. Kinetic data suggested that ATP had its effect by binding to a non-active site in the enzyme to which NAD^+ may also bind. Glutamate, at concentrations below 5 mM, L-alanine and L-aspartate alone or in combination activated reductive amination, but not oxidative deamination, to a maximum of 33·3%. Double reciprocal plots of velocity against either NAD^+ or NADH or glutamate were biphasic. R_s and K_m values, and Hill coefficients calculated from initial velocity data were consistent with a system which exhibits negative cooperativity with respect to binding of substrates and positive cooperativity with respect to catalytic activity. Various aspects of the metabolism of germinating fatty seeds are discussed in relation to this and other work recently reported.

INTRODUCTION

GLUTAMIC dehydrogenases (GDH) have been isolated from a number of plants,[1–3] animal tissues[4] and microorganisms.[5] The influence of a wide variety of metabolic effectors on GDH from animals, notably beef and ox livers, and microorganisms have been studied in detail by Frieden *et al.*,[4,6–14] LeJohn *et al.*,[5,15–19] Sanwal *et al.*[1,20] and Tomkins *et al.*[21,22]

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There are no comparable reports which emphasize the kinetic properties of similar enzymes from higher plant sources, although a recent article[23] described the effect of sulphhydryl group and metal ion inhibitors on a GDH from pea seedlings.

Experiments reported here using extracts from green soybean cotyledons were designed to isolate and purify a glutamate dehydrogenase from a higher plant source and to survey some of its properties, including the influence of metabolic effectors on its activity. Wherever pertinent, comparisons are made with similar enzymes from other sources.

RESULTS

General Properties

Regardless of whether crude or partially purified enzyme extracts were used, only one band with GDH activity was detected when proteins were separated by acrylamide gel electrophoresis and specifically stained. The partially purified enzyme was specific for NAD^+ and NADH (E.C. 1.4.1.2) and had maximum activity at pH 8 in the direction of glutamate formation and at pH 9·3 in the forward direction (Fig. 1).

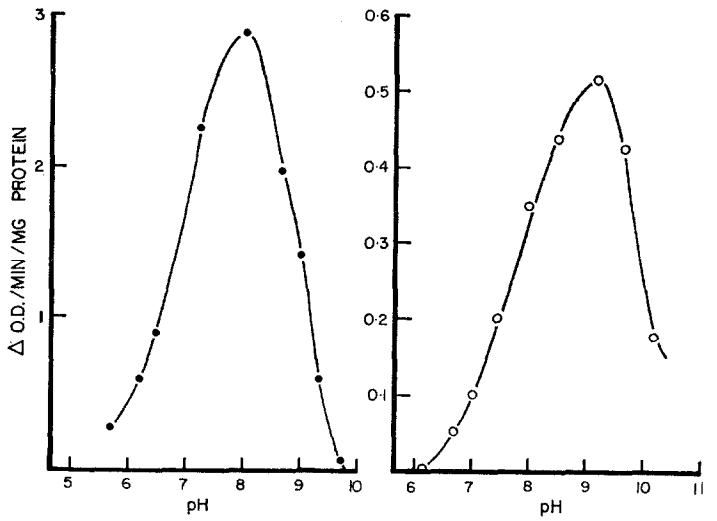

FIG. 1. EFFECT OF pH ON GLUTAMATE DEHYDROGENASE ACTIVITY.
Reductive amination (●); oxidative deamination (○). Assay system: see Methods.

Order of Substrate Addition

Using initial velocity methods similar to those described by Frieden[8] and LeJohn *et al.*[15,19] and product inhibition studies after Cleland,[24] the sequence of addition of substrates to GDH was determined.

With high and constant concentrations of 2-oxoglutarate, double reciprocal plots of initial velocity against NADH at different constant concentrations of ammonia gave parallel lines (Fig. 2). The implication is that the Michaelis constant for NADH is dependent on the concentration of ammonium ions.[25] At high and constant concentrations of

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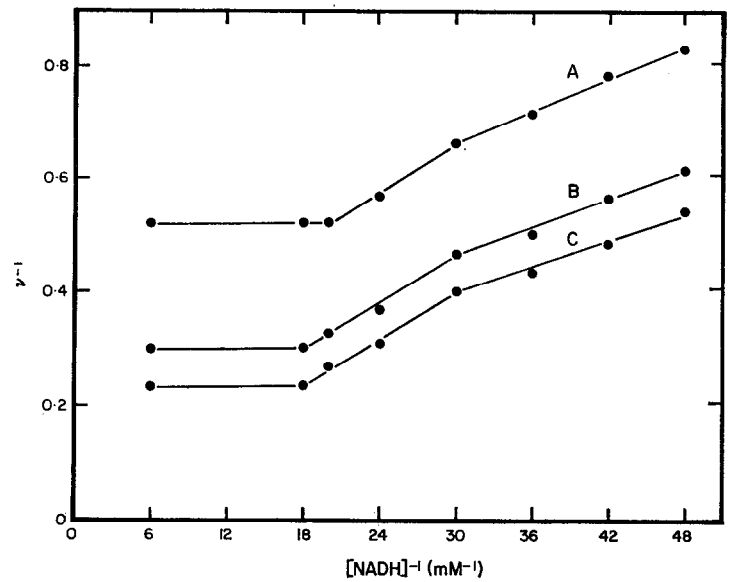


FIG. 2. DOUBLE RECIPROCAL PLOTS OF VELOCITY AGAINST NADH CONCENTRATION IN THE PRESENCE OF CONSTANT SATURATING CONCENTRATION OF 2-OXOGLUTARATE (20 mM) WITH AMMONIA HELD AT FIXED LEVELS.

The concentrations of ammonia used were: A, 10 mM; B, 30 mM and C, 50 mM. Assays were carried out in 0·2 M Tris-HCl buffer, pH 7·75. Enzyme, 125 μg protein.

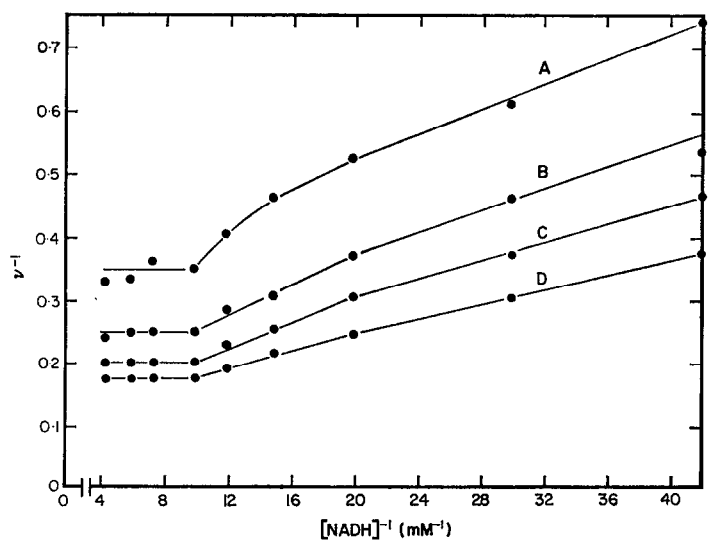


FIG. 3. DOUBLE RECIPROCAL PLOTS OF VELOCITY AGAINST NADH CONCENTRATION IN THE PRESENCE OF SATURATING CONCENTRATIONS OF AMMONIA (50 mM), WITH 2-OXOGLUTARATE HELD AT VARIOUS FIXED LEVELS.

Concentrations of 2-oxoglutarate used were: A, 0·833 mM; B, 1·67 mM; C, 3·33 mM and D, 6·67 mM. Assays were performed in 0·2 M Tris-HCl buffer, pH 8·0. Enzyme, 74 μg protein.

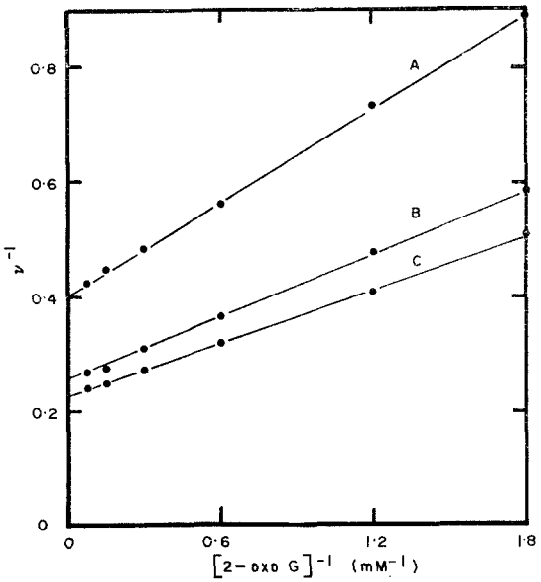


FIG. 4. DOUBLE RECIPROCAL PLOTS OF VELOCITY AGAINST 2-OXOGLUTARATE CONCENTRATION IN THE PRESENCE OF HIGH (0·167 mM), CONSTANT CONCENTRATION OF NADH.

The concentrations of ammonia used were: A, 10 mM; B, 30 mM and C, 50 mM. Assays were carried out in 0·2 M Tris-HCl buffer, pH 8·0. Enzyme, 125 μg protein.

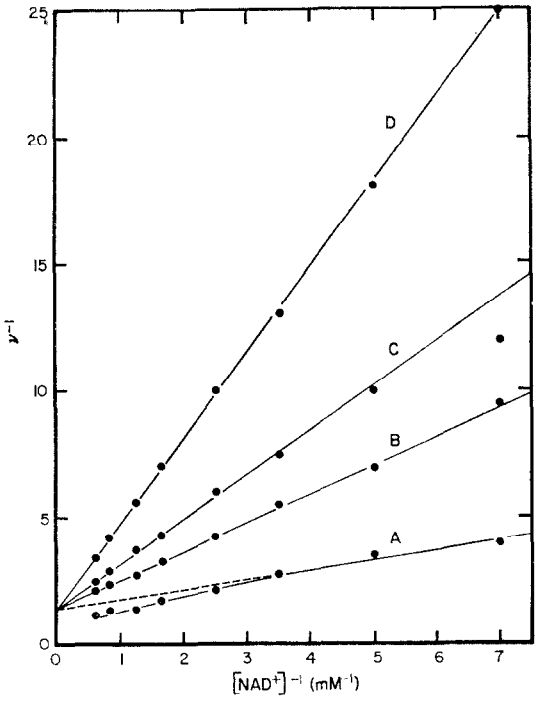


FIG. 5. PRODUCT INHIBITION OF GLUTAMATE DEHYDROGENASE BY NADH, WITH NAD^+ AS THE VARIED SUBSTRATE.

Glutamate was held at a high concentration of 33·33 mM. The concentrations of NADH were: A, 0 mM; B, 0·025 mM; C, 0·05 mM and D, 0·1 mM. Assays were carried out in 0·2 M Tris-HCl buffer, pH 9·2. Enzyme, 100 μg protein.

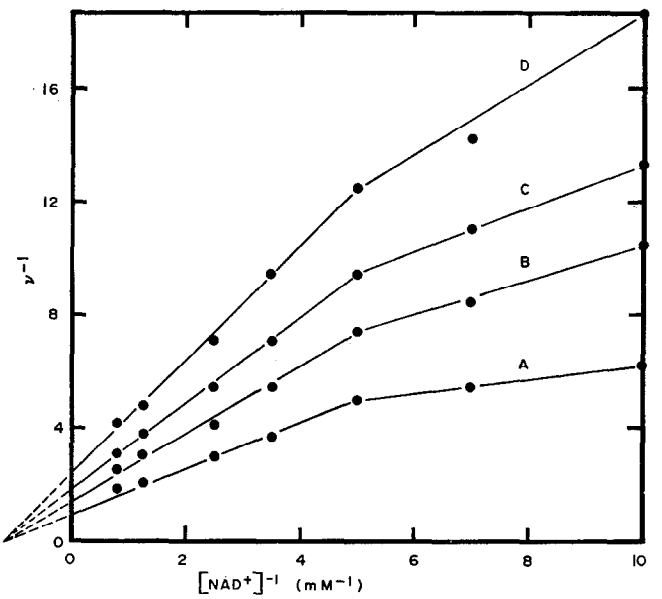
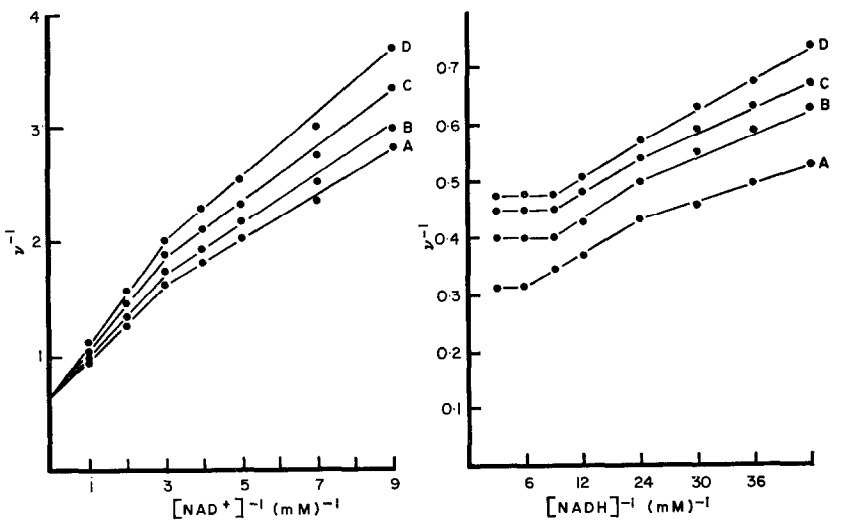


FIG. 6. PRODUCT INHIBITION OF GLUTAMATE DEHYDROGENASE BY AMMONIA, WITH NAD^+ AS THE VARIED SUBSTRATE.

Glutamate was held at a high and constant concentration of 33·33 mM. Concentrations of ammonia used were: A, 0 mM; B, 5 mM; C, 10 mM and D, 20 mM. Assays were conducted in 0·2 M Tris-HCl buffer, pH 9·2. Enzyme, 168 μg protein.



FIGS. 7 and 8. INHIBITION BY ATP.

Assay systems: Fig. 7, Tris-HCl buffer, pH 9·2, 200 mM; glutamate, 50 mM; enzyme protein, 270 μg. Fig. 8, Tris-HCl buffer, pH 8·0, 200 mM; 2-oxoglutarate, 6·67 mM; $(NH_4)_2SO_4$, 50 mM; enzyme protein, 100 μg. The concentrations of ATP used were: A, 0 mM; B, 1 mM; C, 2 mM; D, 3 mM.

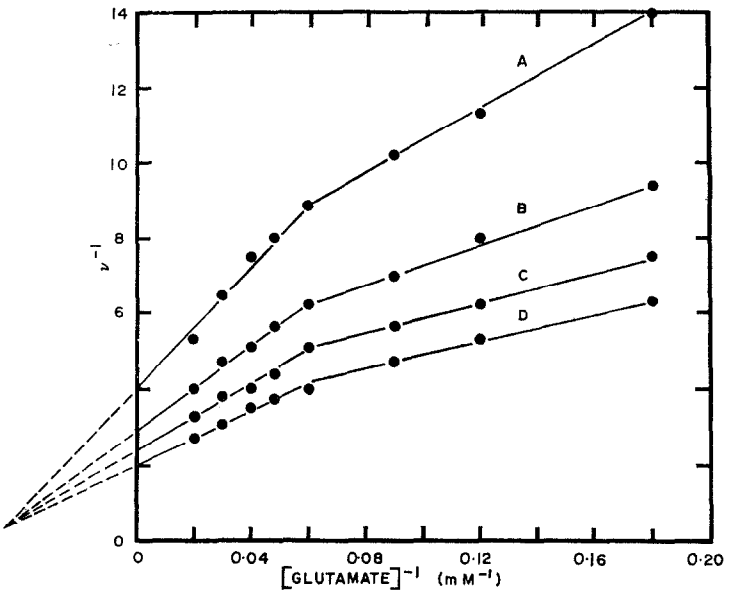


FIG. 9. DOUBLE RECIPROCAL PLOTS OF VELOCITY AGAINST GLUTAMATE CONCENTRATION WITH NAD^+ AT FOUR FIXED LEVELS OF A, 0·29 mM; B, 0·6 mM; C, 0·8 mM; D, 1·2 mM.
Assays were conducted in Tris-HCl buffer, pH 9·2. Enzyme, 150 μg protein.

ammonia, the double reciprocal plots of velocity against NADH, with 2-oxoglutarate held at different levels, would intersect each other at the abscissa if extrapolated (Fig. 3). This showed that the Michaelis constant for NADH was independent of the 2-oxoglutarate concentration. These results suggest that 2-oxoglutarate is the second substrate that binds to the enzyme and are similar to those of LeJohn *et al.*[15,19] who used enzymes isolated from microorganisms. With high, saturating levels of NADH and varying levels of ammonia, the plots of reciprocal of velocity against reciprocal of 2-oxoglutarate showed that the Michaelis constant for 2-oxoglutarate was unchanged and therefore independent of ammonium ion concentration (Fig. 4). The biphasic nature of plots noted here (Figs. 2 and 3) and elsewhere (Figs. 5–9) will be discussed later. Inhibition at high concentrations of NADH were also noted in Figs. 2 and 3 and elsewhere (Fig. 8).

To determine whether NADH was the first substrate to bind to the enzyme, product inhibition studies were performed as suggested by Cleland.[24] In the presence of high concentrations of glutamate, double reciprocal plots of initial velocity against NAD^+ with NADH as the inhibitor showed competitive inhibition (Fig. 5). A biphasic curve was obtained in the absence of inhibitor (Fig. 5, line A) but not in its presence. With ammonia as inhibitor, the double reciprocal plots were noncompetitive (Fig. 6).

These data suggest that the soybean GDH has the same sequence of substrate addition as the NAD-dependent GDH isolated from microorganisms, namely, NADH followed by 2-oxoglutarate and finally ammonia.

Inhibition by Chelating Agents

The addition of either disodium ethylenediamine tetraacetate (EDTA) or 8-hydroxyquinoline to reaction mixtures in various concentrations caused inhibition of GDH activity

(Table 1). EDTA had a greater effect upon reductive amination than upon oxidative deamination. High concentrations of EDTA showed a lower inhibition of both the forward and the reverse reactions (Table 1). High concentrations of 8-hydroxyquinoline were required for inhibition of reductive amination (Table 1). These results were similar to those obtained for the pea GDH isolated by Yamasaki and Susuki.[23]

TABLE 1. EFFECT OF CHELATING AGENTS ON GLUTAMATE DEHYDROGENASE ACTIVITY

Inhibitor	Final concentration (mM)	Inhibition (%)	
		NAD reduction	NADH oxidation
Disodium ethylenediamine Tetraacetate	0·005	0	12·2
	0·007	0	22
	0·01	0	51·2
	0·1	0	61
	1·0	30	61
	10	30	41·6
	100(40)	20	(30·5)
8-Hydroxyquinoline	0·1		0
	0·5		16·7
	1·0		27·1
	5·0		68·8
	7·0		79

ATP Inhibition

At concentrations of 1 mM and above ATP inhibited GDH activity in both directions (Figs. 7, 8). In both cases, double reciprocal plots of initial velocity against low coenzyme concentrations in the presence of 0, 1, 2 and 3 mM ATP gave a family of lines which intersected to the left of the ordinate. As either NAD^+ or NADH concentrations were raised, biphasic plots were evident. Increasing NAD^+ concentration competitively eliminated ATP inhibition (Fig. 7) whilst the biphasic effect caused by increasing reduced coenzyme was uncompetitive with ATP inhibition (Fig. 8). GTP also slightly inhibited the enzyme but AMP, ADP and GMP had no effect.

Amino Acid Activation

L-Arginine, L-glutamine, L-serine, citrate and DL-isocitrate had no effect on GDH activity when added to assay mixtures in concentrations as high as 30 mM. L-Glutamate increased the rate of reductive amination to a slight (7%) but reproducible degree at concentrations of 5 mM and below (Table 2). Above this concentration glutamate was a product inhibitor. L-Alanine and L-aspartate activated reductive amination, but not oxidative deamination, to maxima of 11·1 and 33·3%, respectively (Table 2). When any two or all three of these amino acids were added together, their combined activating effect was additive up to a maximum of 33·3%.

Michaelis Constants and Analysis of Biphasic Plots

Biphasic double reciprocal plots were recorded for glutamate (Fig. 9) in addition to those noted earlier for NAD^+ and NADH but not for 2-oxoglutarate or ammonia. Apparent K_m values were calculated from these plots and are listed in Table 3 along with values

TABLE 2. ACTIVATION OF GLUTAMATE DEHYDROGENASE BY AMINO ACIDS

Addition	Conc. (mM)	Activation of Reductive Amination (%)
L-glutamate	3·5	5·6
L-glutamate	5·0	7
L-alanine	10·0	5·6
L-alanine	20·0	11·1
L-alanine	30·0	11·1
L-aspartate	10·0	20
L-aspartate	20·0	33·3
L-aspartate	30·0	33·3
L-alanine + L-aspartate	10·0 of each	25·9
L-alanine + L-aspartate	20·0 of each	33·3
L-alanine + L-aspartate + L-glutamate	10·0:10·0:3·5	29·6

obtained by other workers for glutamate dehydrogenases from corn leaves,[26] *Blastocladiella*[16] and beef liver.[6,8] Some K_m values are designated K_m^L and K_m^H after Datta and Gest who reported a similar phenomenon for homoserine dehydrogenase from *Rhodospirillum rubrum*.[27]

Hill[28] plots for NAD^+ (data from Fig. 6A), NADH (Fig. 8A) and glutamate (Fig. 9B) were constructed and an example of these is contained in Fig. 10. It can be seen that the slope of the line (Fig. 10) varies with NAD^+ concentration. At very low levels of NAD^+, the slope varies from 1·0 to 0·75 and then decreases to 0·52 in the region of transition from low to high enzymic activity (i.e. 0·2 mM NAD^+) and then increases to 1·0 at high NAD^+

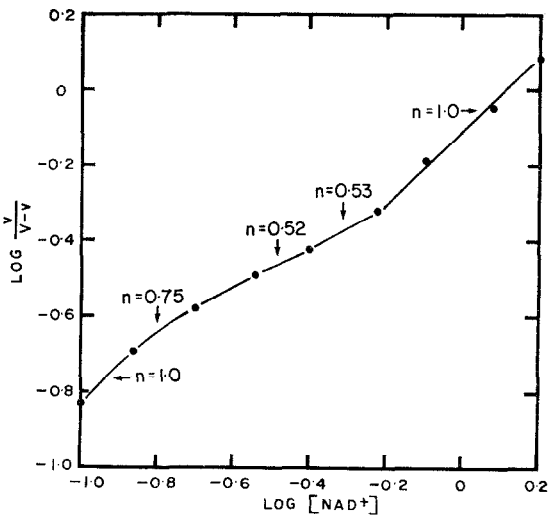


FIG. 10. HILL PLOT FOR THE DATA PRESENTED IN FIG. 6A.

[26] W. A. BULEN, *Arch. Biochem. Biophys.* **62**, 173 (1956).
[27] P. DATTA and H. GEST, *J. Biol. Chem.* **240**, 3023 (1965).
[28] A. J. HILL, *Biochem. J.* **7**, 471 (1913).

concentrations. Nearly identical results obtained for NADH and glutamate are not reported.

R_s values (i.e. the ratio of the NAD^+, NADH or glutamate concentration at 90 and 10 per cent of the saturation velocity), calculated from the same data used to calculate Hill coefficients, far exceeded the value of 81 which is expected for a classical Michaelis–Menten hyperbola[29] (Table 3).

TABLE 3. APPARENT MICHAELIS CONSTANTS AND R_s VALUES FOR GLUTAMATE DEHYDROGENASES

		K_m(M)				
Substrate		Soybean cotyledons	Corn leaves[26]	*Blastocladiella*[16]	Beef liver[6,8]	Soybean GDH R_s values
2-Oxoglutarate		$1{\cdot}2 \times 10^{-3}$	$1{\cdot}5 \times 10^{-3}$	$5{\cdot}6 \times 10^{-4}$	7×10^{-4}	
$(NH_4)_2SO_4$		$0{\cdot}94 \times 10^{-2}$	$1{\cdot}01 \times 10^{-2}$	$4{\cdot}0 \times 10^{-2}$	$3{\cdot}2 \times 10^{-3}$	
NADH	L	$1{\cdot}5 \times 10^{-5}$	$3{\cdot}65 \times 10^{-5}$	$3{\cdot}3 \times 10^{-5}$	$1{\cdot}15 \times 10^{-4}$	
	H	$3{\cdot}9 \times 10^{-5}$	—	—	—	160
Glutamate	L	$8{\cdot}0 \times 10^{-3}$	—	$1{\cdot}43 \times 10^{-3}$	$1{\cdot}8 \times 10^{-3}$	
	H	$2{\cdot}5 \times 10^{-2}$	—	$1{\cdot}33 \times 10^{-2}$	—	171
NAD^+	L	$2{\cdot}1 \times 10^{-4}$	—	$4{\cdot}0 \times 10^{-4}$	$7{\cdot}0 \times 10^{-5}$	
	H	$1{\cdot}2 \times 10^{-3}$	—	$2{\cdot}0 \times 10^{-3}$	$2{\cdot}0 \times 10^{-3}$	176

Apparent Michaelis constants at low and high substrate concentrations are designated K_m^L and K_m^H, respectively, after Datta and Gest.[2]

For all of those biphasic curves used for Hill plot and R_s value determinations and for most of the other biphasic curves the difference between two linear regressions was shown to be significant at $P = 0{\cdot}05$ (see Experimental).

DISCUSSION

Purification data (see Experimental) suggest that in soybean cotyledons, GDH activity is localized in mitochondria as it is in many other tissues.[2,30,31] 70% of GDH activity was found in the 'particulate fraction'. At least some of the remaining 30% activity found in the 'soluble fraction' may also have been leached from mitochondria during extraction.

Certain of the properties of soybean GDH closely resembled those of similar enzymes from other sources. The observed optimum of pH 8 in the direction of glutamate formation is the same as that of the NAD-specific enzyme from *Thiobacillus*[5] and *Neurospora*[32] but higher than those of *Blastocladiella* (pH 7)[16] and pea seedlings (pH 7·5).[23] The pH optimum of 9·3 in the forward direction is slightly lower than those of *Thiobacillus* (pH 9·5)[5] and pea seedlings (pH 10)[23] but higher than those of *Blastocladiella* (pH 8)[16] and beef liver enzyme (pH 8·5).[33] Experiments with the chelators EDTA and 8-hydroxyquinoline suggest that it is a metalloenzyme similar to that found in peas and in other organisms.[23] Its K_m values (Table 3) were comparable to those of enzymes from corn leaves, *Blastocladiella*

[29] A. LEVITSKI and D. E. KOSHLAND, *Proc. Natl Acad. Sci. U.S.* **62,** 1121 (1969).
[30] G. H. HOGEBOOM and W. C. SCHNEIDER, *J. Biol. Chem.* **204,** 233 (1953).
[31] J. VAN DIE, *Acta Bot. Neerland* **11,** 1 (1962).
[32] C. S. STACHOW and B. D. SANWAL, *Biochim. Biophys. Acta* **139,** 294 (1967).
[33] J. WOLFF, *J. Biol. Chem.* **237,** 230 (1962).

and beef liver. Finally, the sequential order of substrate addition was the same as that found for similar enzymes from microorganisms. Enzyme preparations from beef liver,[8] frog liver[34] and dogfish[35] catalyse reactions in which the coenzyme (NADH or NADPH) binds first to the enzyme and NH_4^+ binding precedes 2-oxoglutarate. In *Thiobacillus novellus*[15] the NADP-specific enzyme has the same order of substrate addition as that of bovine liver enzyme,[8] whereas the NAD-specific enzyme from *Thiobacillus* as well as that from *Blastocladiella*[19] binds 2-oxoglutarate before NH_4^+. The soybean GDH would seem to be typical of NAD-specific enzymes.

The order of substrate addition to soybean GDH was determined using similar methods to those described by Frieden[8] and LeJohn *et al.*[15,19] and product inhibition studies after Cleland.[24] The parallel double reciprocal plots in Fig. 2 indicate an irreversible step between the addition of NADH and ammonia to the enzyme.[25] The irreversibility is caused by the presence of an infinitely high concentration of 2-oxoglutarate which suggests that the keto acid occurs between NADH and ammonia in the reaction sequence. The Michaelis constant for NADH is dependent upon the concentration of ammonium ion (Fig. 2). On the other hand, the double reciprocal plots of varying NADH concentrations at fixed levels of 2-oxoglutarate and saturating concentrations of ammonia (Fig. 3) or of varying 2-oxoglutarate at fixed levels of ammonium ion and high levels of NADH (Fig. 4) intersect on the abscissa if extrapolated. These data indicate that at high, constant levels of either ammonia (Fig. 3) or NADH (Fig. 4), the Michaelis constants of the variable substrates are independent of the concentrations of the other substrates held at fixed levels and irreversible steps do not occur. Analysis of such data by the steady state method after Frieden[8] confirms that the second substrate to bind to the enzyme is 2-oxoglutarate. To ascertain whether NADH or ammonia is the first substrate to bind to the enzyme, product inhibition studies after Cleland[24] were performed. When NADH was the inhibitor, competitive inhibition was observed with the variable substrate NAD^+ (Fig. 5). The conclusion is that the inhibitor combines with the same enzyme form as does the variable substrate and that saturation with the variable substrate overcomes this inhibition. When ammonia was the inhibitor (Fig. 6), non-competitive inhibition was observed with NAD^+ as the variable substrate, a result which indicates that the inhibitor combined with a different enzyme form from that with which the variable substrate combined. The conclusion from data in Figs. 5 and 6 is that NAD^+ and NADH, but not ammonia, can bind to the free enzyme.

Concentrations of 1, 2 and 3 mM ATP inhibited soybean GDH 13, 18 and 22% respectively. Other purine nucleotides were without significant effect. A number of nucleotides have been tested for their effects on the initial velocity of GDH. AMP and ADP activated beef liver GDH using any coenzyme species whilst ATP inhibited NADH oxidation, activated NAD^+ and $NADP^+$ reduction and had little effect upon NADPH oxidation.[4,17] Guanosine nucleotides (GDP and GTP) are strong inhibitors of beef liver GDH using any coenzyme species.[10,33] All purine nucleotides, whether they activate or inhibit the reaction, appear to attach to the same specific purine nucleotide site on the bovine liver enzyme.[10] From his studies of purine nucleotide and coenzyme binding to beef liver GDH, Frieden[10,36] postulated three distinct and specific binding sites as follows; (a) an active site which binds oxidized and reduced di- and triphosphopyridine nucleotides, (b) a non-active site specific for purine nucleotides and to which NAD^+ also binds, and

[34] L. A. FAHIEN, B. O. WIGGERT and P. P. COHEN, *J. Biol. Chem.* **240**, 1083 (1965).
[35] L. CORMAN, L. M. PRESCOTT and N. O. KAPLAN, *J. Biol. Chem.* **242**, 1383 (1967).
[36] C. FRIEDEN, *J. Biol. Chem.* **238**, 146 (1963).

(c) a non-active site which binds NADH. The NAD-specific enzyme from *Neurospora crassa*[20] can be inhibited by a number of purine nucleotides including AMP, GMP and GTP. Studies of the NAD-specific GDH from *Blastocladiella emersonii*[16–19] have shown that ATP inhibits the reaction in both directions of assay, whereas AMP activates. The effects of NAD^+ and NADH on ATP inhibition in the case of soybean GDH (Figs. 7 and 8) suggest that ATP may bind to a site on the enzyme other than the active site although, since the concentrations of ATP used were high (up to 3 mM), this site may not be effective in controlling enzyme activity under normal physiological conditions. Increasing NAD^+ concentration (Fig. 7) competitively eliminated ATP inhibition, which could mean either that ATP had its inhibitory effect by binding to the active site and was displaced by NAD^+ or that ATP bound to a non-active site from which it was displaced either by further binding of NAD^+ to the active site (causing, for instance, protein conformation changes which displaced ATP) or by competition of NAD^+ with ATP for the non-active site. In view of Frieden's earlier work with beef liver GDH[10,36] it is possible that the soybean enzyme has a non-active purine nucleotide-specific site to which NAD^+ also binds. This conclusion is strengthened by the fact that NADH did not displace ATP. The uncompetitive relationship between the reduced coenzyme and ATP would not occur if ATP bound to the active site (Fig. 8).

As mentioned earlier, this purine nucleotide site may not be physiologically significant. The high concentrations of ATP which have to be used to obtain inhibition suggest that the soybean enzyme may exhibit only 'residual allosterism'. It is possible that the higher plant GDH has evolved from an ancestral enzyme more like the microorganismic GDH species, which are more sensitive to purine nucleotides. It may also be that purine nucleotide control of GDH assumed progressively less importance in the slower metabolism characteristic of higher plant cells. The removal of natural selection pressure from this facet of GDH activity would have allowed changes to occur in the amino acid constitution of the enzyme which would not necessarily favour continued high specificity of the purine nucleotide site. Hence, the site must be 'loaded up' with nucleotide before it exhibits characteristics typical of similar enzymes from other sources. This phenomenon might be termed 'residual allosterism'.

Whether soybean GDH contains a non-active site to which NADH also binds is not demonstrated by our kinetic data.

L-Alanine, L-aspartate and L-glutamate activated reductive amination but had no effect upon oxidative deamination (Table 3). Hershko and Kindler[37] showed that amino acid effectors do not bind to the active site or to the purine nucleotide site of ox liver GDH but share a common amino acid site. This may also be true for soybean GDH. The activating effect produced by amino acids in soybean contrasted with inhibition by amino acids of a NAD-specific GDH from plant leaves, reported by Sims *et al.*[38]

Double reciprocal plots of initial velocities against either NAD^+ (Figs. 5–7) or NADH (Figs. 2, 3 and 8) or glutamate (Fig. 9) were usually biphasic downwards. R_s values calculated from these data (Table 3) were much greater than 81, which is the expected value for a typical Michaelis–Menten hyperbola, and Hill coefficients calculated from the same data were less than unity, which is also the expected value in normal Michaelis–Menten kinetics. Deviations from linearity of Lineweaver–Burk plots of reaction activity have been

[37] A. Hershko and S. H. Kindler, *Biochem. J.* **101**, 661 (1966).
[38] A. P. Sims, B. F. Folkes and A. H. Bussey, in *Recent Aspects of Nitrogen Metabolism in Plants*, Academic Press, London (1968).

observed with GDH from various sources. In all cases, high concentrations of NAD^+ resulted in activation of the enzyme. Bisphasic NAD^+ activation of beef liver GDH was attributed to coenzyme binding at an allosteric site.[7,36] High concentrations of NADH[6,16,39,40] and NADPH[20,39,41,42] result in inhibition, a phenomenon also observed with soybean GDH. Deviaton from linearity has also been observed for $NADP^+$ activation of ox liver GDH.[43] Engel and Dalziel[43] explained that the tri- or tetraphasis kinetic behaviour which they observed was identical to that predicted by Levitski and Koshland[29] for a system in which there is negative cooperativity with respect to binding and positive cooperativity with respect to catalysis. Monod *et al.*[44] had postulated earlier that allosteric systems appeared to be always positive or cooperative. Their model can account for the characteristic sigmoid rate plot which corresponds to a double reciprocal plot that is concave upwards, but does not explain the phenomenon observed in studies of soybean GDH (i.e. biphasic downwards). On the other hand, the model for allosteric proteins forwarded by Koshland *et al.*[45] is symmetrical in that interactions between enzymes and ligands may be negative or positive, i.e. ligand binding at one site may either increase or decrease the ligand affinity of remaining sites on an enzyme with more than one active centre per molecule. When positive cooperativity with respect to binding is exhibited, the double reciprocal plot of initial velocity against substrate concentration is concave upwards, R_s values are less than 81 and Hill coefficients are greater than unity, whereas for negative cooperativity, double reciprocal plots are biphasic or concave downwards, R_s values are greater than 81 and Hill coefficients are less than unity.[29] Data reported here for soybean GDH favour Koshland's model and demonstrate negative cooperativity with respect to binding and positive cooperativity with respect to catalysis. The effect of the latter phenomenon is as if the number of binding sites increases with the substrate concentration, successive sites having larger Michaelis constants (Table 3, K_m^H values) for the substrates and perhaps greater turnover rates.[43] The data in Fig. 3, line A, a Lineweaver–Burk plot that is concave downwards, are particularly characteristic of a negatively cooperative system where sequential binding of ligands leads to several changes in affinity towards substrates at the active site.[45] Levitski and Koshland suggest that negative cooperativity in many enzymes has been indicated in the literature but not recognized and they cite several examples.[29] Apart from their own observations with soybean GDH, these authors have found only one other example in the literature of what might be a similar phenomenon in a higher plant enzyme. Bryan[46] reports biphasic double reciprocal plots for homoserine dehydrogenase isolated from *Zea mays* roots.

The authors have speculated on the physiological significance of the observed kinetic properties of soybean GDH. Whereas the effect of positive interactions is to make an enzyme very sensitive to small changes of substrate or coenzyme concentration over a narrow range, the effect of negative interactions is to make the reaction rate continuously responsive to concentration changes over a wide range.[43] Negative cooperativity, then, insulates an enzyme against changes in metabolite concentration.[47] During senescence in

[39] L. A. FAHIEN, B. O. WIGGERT and P. P. COHEN, *J. Biol. Chem.* **240**, 1091 (1965).
[40] L. CORMAN and N. O. KAPLAN, *J. Biol. Chem.* **242**, 2840 (1967).
[41] S. GRISOLIA, C. L. QUIJADA and M. FERNANDEZ, *Biochim. Biophys. Acta* **81**, 61 (1964).
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[43] P. C. ENGEL and K. DALZIEL, *Biochem. J.* **115**, 621 (1969).
[44] J. MONOD, J. WYMAN and J. P. CHANGEUX, *J. Mol. Biol.* **12**, 88 (1965).
[45] D. E. KOSHLAND, G. NEMETHY and D. FILMER, *Biochemistry* **5**, 365 (1966).
[46] J. K. BRYAN, *Biochim. Biophys. Acta* **171**, 205 (1969).
[47] A. CONWAY and D. E. KOSHLAND, *Biochemistry* **7**, 4011 (1968).

tissues such as cotyledons and endosperm, rapid hydrolysis of proteins leads to the accumulation of amino acids including glutamate.[48] It is also known that cotyledons contain throughout their limited life-spans larger quantities of NAD^+ and relatively less NADH than other tissues.[49] From other studies with castor bean endosperm it is also known that protein hydrolysis is linked through transamination and deamination to gluconeogenesis.[50] The ammonia released is utilized to produce the translocate, glutamine.[50] In fatty soybean cotyledons events comparable to those reported for castor bean endosperm may occur. Desensitization of GDH by accumulating NAD^+ and glutamate during cotyledon senescence may allow glutamine synthetase to compete more effectively with GDH for available glutamate. However, positive cooperativity of GDH with respect to catalysis would be apparent in the presence of high levels of NAD^+ and glutamate thus ensuring the continued breakdown of glutamate with the attendant production of ammonia for glutamine synthesis and 2-oxoglutarate for the gluconeogenic process. In addition, the cotyledon GDH probably maintains a biosynthetic role for the continued formation of glutamate for protein synthesis since the rate at which it catalyzes reductive amination is several times greater than the rate for oxidative deamination. Activation of GDH unidirectionally by L-alanine, L-aspartate and L-glutamate may aid in this process.

The observed kinetic properties of soybean GDH may be important in glutamine synthesis, gluconeogenesis and amino acid synthesis in the cotyledon.

EXPERIMENTAL

Plant Material

Seeds of *Glycine max* L. var. Canadian No. 1 were mixed with "Terrachlor" (Olin Matheson Chemical Corp., Little Rock, Arkansas), a dry powdered soil fungicide, and germinated in moist sand in the greenhouse in a 16 hr light regime for 11 days.

Chemicals

All general chemicals were of the highest purity available commercially. Fine chemicals were purchased from Calbiochem., Los Angeles, California, Nutritional Biochemicals Corp., Cleveland, Ohio and Sigma Chemical Co., St. Louis, Missouri.

Enzyme Extraction and Partial Purification

All procedures were performed at 4°. Cotyledons were macerated in a Waring blendor for 1–2 min with 2 ml/g of a solution containing 0·4 M sucrose, 0·2 M Tris, 0·1 M K_2HPO_4 and 0·2 M sodium citrate, pH 7·9. The macerate was squeezed through 4 layers of cheesecloth and the filtrate centrifuged at 1000 *g* for 10 min to remove chloroplasts.[51] A 5% aliquot of the supernatant was recentrifuged for 30 min at 10,000 *g*. The GDH activity of the supernatant obtained after the second centrifugation, referred to henceforth as the 'soluble fraction', was determined. The pellet, containing largely mitochondria as judged by microscopic inspection under oil immersion, was resuspended in 0·2 M Tris-HCl buffer, pH 8·0, frozen and thawed twice and ground for 20 min in a mortar with acid washed sand. After removal of sand and organelle debris by centrifugation at 49,000 *g* for 15 min, GDH activity was determined in this 'particulate fraction'. Maximum GDH activity was released from mitochondria by this treatment as attempts to detect residual enzyme activity in the organelle debris mentioned above were unsuccessful. 70% of GDH activity was found in the 'particulate fraction' and 30% in the 'soluble fraction' which when combined correspond to 'crude extract' in Table 4.

The remaining 95% of the filtrate, after straining the crude cotyledon homogenate through cheesecloth, was treated as above except that the final 'particulate fraction' was not combined with the 'soluble fraction'. Instead, it was first washed with half-strength maceration medium and then to it was added solid $(NH_4)_2SO_4$ to 43% saturation with stirring over a 30 min period. Precipitated protein was removed by

[48] A. C. CHIBNALL, in *Protein metabolism in Plants*, Yale University Press, New Haven, Connecticut (1939).
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[51] W. S. PIERPOINT, *Biochem. J.* **71**, 518 (1959).

centrifugation and a further quantity of $(NH_4)_2SO_4$ added over a 30 min period with stirring to 53% saturation. The 43–53% $(NH_4)_2SO_4$ protein fraction, collected by centrifugation, was resuspended in 0·2 M Tris-HCl buffer, pH 8·0 and dialysed overnight against 0·01 M Tris-HCl at the same pH. After centrifugation of the dialysate at 20,000 *g* for 15 min, to remove precipitate formed during dialysis, a purification of 250-fold was obtained in the supernatant (Table 4).

TABLE 4. PURIFICATION OF GLUTAMATE DEHYDROGENASE FROM SOYBEAN COTYLEDONS

Fraction	Volume (ml)	Protein (mg/ml)	Specific activity (enzyme units*/ mg protein)	Purification	Recovery (%)
Crude dialyzed extract	684	20	1·6	1	100
Particulate extract	100	3·2	50	31	73·9
43–53% $(NH_4)_2SO_4$	14	0·78	400	250	20·2

* A unit of enzyme is defined as the amount that causes a decrease of 0·01 in optical density in 1 min.

Enzyme Assays

Soybean GDH was assayed by measuring the initial rate of oxidation of NADH or reduction of NAD^+ in 3 ml silica cuvettes at 340 nm in a Unicam 800 automatic recording spectrophotometer. The standard assay systems were as follows: in the case of NADH oxidation, either KH_2PO_4–K_2HPO_4 buffer, pH 5·7–8, or K_2HPO_4–K_3PO_4 buffer, pH 8·5–11, 200 mM; 2-oxoglutarate, 13·3 mM; $(NH_4)_2SO_4$ or NH_4Cl, 50 mM; NADH, 0·22 mM; enzyme protein, 100–200 μg. In the case of NAD^+ reduction, buffer as above, 200 mM; sodium glutamate, 33·3 mM; NAD^+, 1·0 mM; enzyme protein, 200–300 μg. When other assay systems were used they are specified in the text. The reproducibility of initial rates was generally ± 5%. Protein was estimated by the method of Lowry *et al.*[52] using crystalline bovine serum albumin as standard. Disc electrophoresis was performed at 4° by the procedure described by Davis.[53]

Statistical Analysis of Biphasic Initial Velocity Plots

Initial velocity data were plotted in the reciprocal form ($1/v$) against reciprocal substrate concentration ($1/S$). When a biphasic curve was obtained from these plots, the two regression coefficients were computed and the null hypothesis, i.e. whether or not the two regression coefficients could be considered as estimates of a common population regression coefficient, was tested by '*t*' and '*F*' values.[54] The significance of the difference between the two regressions of each curve was determined at the 5% level of significance using Fisher's table of *t*-values and Pearson's table of *F*-values.[54] Data analyses were performed with an Olivetti–Underwood Programma 101 Computer.

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