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Glutamate Dehydrogenase in Higher Plants
V. Purification and Properties of the Enzyme from Turnip Roots.

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Summary

Glutamate dehydrogenase (GDH) was isolated from turnip roots and purified about 400 fold. The enzyme showed much higher activity in the oxidation reactions of NADH or NADPH than in the reduction reactions of NAD or NADP. Optimum pHs for NADH-, NAD-, NADPH- and NADP-dependent GDH were 8.4, 9.1, 8.0 and 9.0, respectively. The molecular weight was estimated to be about 250,000. The enzyme had high stability for temperature, changes of pHs and storage in deep freezer. On the other hand its activity was inhibited by sulfhydryl reagents and chelating agents. Ag^{+} and Zn^{2+} depressed both NADH- and NADPH- dependent GDH, but NADPH- dependent GDH was activated by Mg^{2+} and Mn^{2+}. Michaelis constants were also estimated.

In higher plants, GDH has been considered to be one of the most important enzymes acting in the incorporation of ammonia into organic nitrogen compounds and is therefore of considerable physiological interest (1). However few detailed biochemical studies on GDH in higher plants have been carried out, and physiological phenomena relating to this enzyme in higher plants have been interpreted by the knowledge obtained from animals or microorganisms. In our previous investigations (2–5), the properties of GDH in higher plants were shown to be considerably different from those in animals or microorganisms.

Thus this paper deals with isolation, purification and some biochemical properties of GDH from turnip roots.

Materials and Methods

Plants Material. Fresh roots of turnip (Brassica Campestris L. subsp. Rapa.) obtained from the local market were used as the source of enzymes throughout this work.

Reagents. DEAE-cellulose was purchased from Brown Company and Sephadex G–200 was from Pharmacia Fine Chemicals. NADH, NAD, NADPH and NADP were purchased from Boehringer Mannheim Company.

Enzyme Assay. GDH activity was measured by the initial rate of reduction or oxidation of coenzyme at 340 mμ at 25°C (4). One unit activity is defined as the amounts of enzyme which cause a decrease or increase of 0.1 in the absorbance at 340 mμ in one minute. Some properties of turnip GDH were measured in comparison with that of beef liver GDH.

Protein Determination. Protein was determined by the colorimetric method of Lowry et al (6) using crystalline bovine serum alubumin as the standard.

Results and Discussions

Purification.

Five hundreds and fourty three Kg of turnip root tissues were stored at –15°C for a week. After thawing at room temperature these materials were homogenized in small batches with one-tenth volume of 0.2 M potassium phosphate buffer (pH 7.8) containing 10^{-3} M β-mercaptoethanol for 5 min in a mixer. The homogenate was packed into calico bags and squeezed by a basket centrifuge. The extract was brought to 70% saturation with solid $(NH_4)_2SO_4$. The precipitate collected with continuous centrifuge, was dissolved in 0.02 M potassium phosphate buffer (pH 7.8) containing 10^{-4} M β-mercaptoethanol (dialyzing buffer) and then dialyzed overnight against the same buffer. The precipitate formed during dialysis was removed by centrifugation and the supernatant was stored at –15°C until all materials were brought to this step.

The combined enzyme solution was added with solid $(NH_4)_2SO_4$ to 30% saturation by stirring over a 30 minute period. Precipitated protein was removed by centrifugation and the supernatant was supplied with further quantity of $(NH_4)_2SO_4$ to a concentration of 50% saturation. The 30–50% $(NH_4)_2SO_4$ protein fraction, collected by centrifugation, was resuspended in dialyzing buffer and dialyzed against the same buffer.

After centrifugation of dyalyzate, the supernatant was brought to 55% saturation with acetone. The 0–55% acetone precipitate was collected, dissolved and dialyzed overnight in the same manner as described above.

The dialyzate was treated with alumina C_γ gel (7) at a concentration of 1 mg/1 mg protein. The suspension was stirred for 10 min and left at 0°C for 3 hours. The gel was recovered by centrifugation and the enzyme was eluted from the gel by stirring with 1 M potassium phosphate buffer (pH 7.8) for 1 hour and again with the same buffer. The combined eluate was added with solid $(NH_4)_2SO_4$ to 30% saturation and the precipitate was dialyzed against 0.025 M Tris-HCl buffer (pH 7.4) containing 10^{-4}M β-mercaptoethanol.

The dialyzed solution was applied to a DEAE-cellulose column (3.8×60 cm). The enzyme was eluted with an increasing concentration of Tris-HCl buffer from 0.025 to 0.2 M; the flow rate was 50 ml per hour. The major enzyme fractions were pooled and treated with $(NH_4)_2SO_4$ at a concentration of 5 g per 10 ml.

The precipitate thus obtained was dissolved in dialyzing buffer, dialyzed and placed on a Sephdaex G–200 column (5.5×120 cm) previously treated with the same buffer. The enzyme was eluted by continuous washing with the same buffer. The major enzyme fractions were collected and used in the following experiments. All operations were carried out below 5°C. A summary of the purification procedure is given in Table 1. Starting with 543 Kg of turnip root tissues, 63 mg of the preparation purified about 400 fold was obtained

TABLE 1. *Purification of GDH from Turnip Roots.*

Purification steps	Total*1 units ($\times 10^3$)	Protein (g)	Specific*1 activity	Degree of*1 purification	Yield*1 (%)
Crude extract	3395	893	3.8	1.0	100
0–70% $(NH_4)_2SO_4$	2081	269	7.8	2.0	61.3
30–50% $(NH_4)_2SO_4$	1176	123	9.5	2.5	34.6
0–55% acetone	603	39.4	15.3	4.0	17.7
Alumina C_γ gel eluate	350	11.6	30.3	8.0	10.3
0–30% $(NH_4)_2SO_4$*2	231	1.18	198.0	52.0	6.8
DEAE-cellulose eluate	103	0.188	547.0	143.9	3.0
Sephadex–G200 eluate	95	0.063	1510.0	397.4	2.7

*1 Data were shown as NADH-dependent GDH activity
*2 Enzyme was dissolved in 1 M K-phosphate buffer

pH Optimum and Substrate Specificity

Fig. 1 shows the pH optimum and relative activity of turnip GDH. Optimum pHs for NADH-, NAD, NADPH- and NADP-dependent GDH were 8.4, 9.1, 8.0 and 9.0 respectively, and relative activity of individual coenzyme dependent GDH at their optimum pHs were 100, 2.78, 10.50 and 0.18. The activity of reductive amination, namely NADH-dependent GDH, appeared to be much higher with the preparation of turnip roots than those of beef liver (8). It may reflect the fact that in higher plants the incorporation of ammonia into glutamate is one of the most important reactions.

Substrate specificity for keto acids and amino acids were examined with α-keto glutarate, pyrvate, L-glutamate, D-glutamate, L-glutamine, DL-nor valine, L-alanine and L-aspartate. Compared with beef liver GDH (10), the turnip GDH were highly specific for α-keto glutarate (aminating system) and L-glutamate (deaminating system).

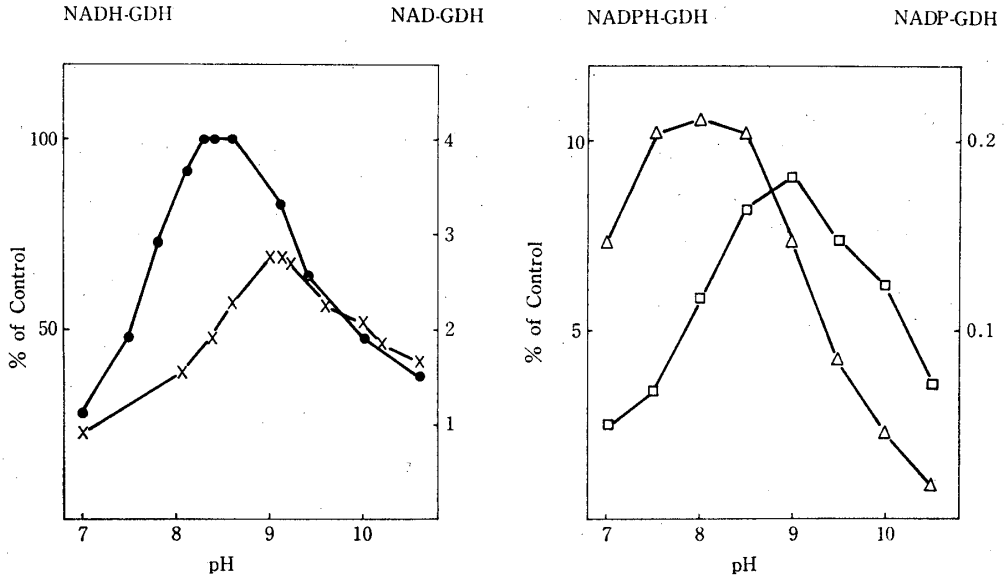

FIG. 1. Effect of pH on GDH activity.*
(●) NADH-GDH, (×) NAD-GDH, (△) NADPH-GDH, (□) NADP-GDH
* Activity is shown as the % of NADH-GDH activity at pH 8.4

Effect of Storage Temperature

As shown in Fig 2. both NADH-and NADPH-dependent GDH from turnip were very stable and the enzyme solution could be stored in a frozen state for at least a month without losing more than 10% of its initial activity. Even at 5°C, the activity of turnip GDH decreased only 30% during a month while that of beef liver was lost almost completely in a period of 5 days.

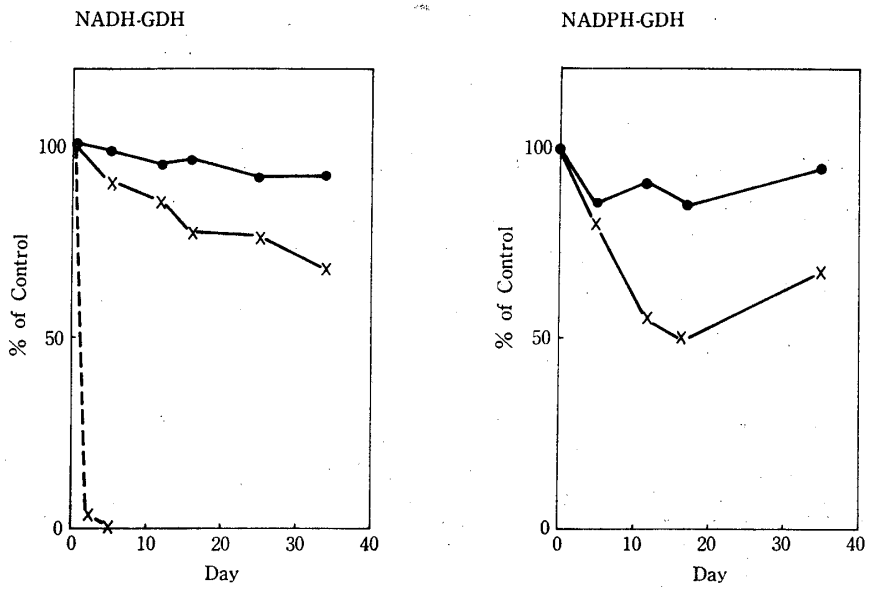

FIG. 2. Effect of storage temperature on GDH activity.
(●) Deep freezer (×) 5°C (×···×) Beef liver GDH

Thermal Inactivation

Fig. 3 shows that turnip GDH is fairly stable to heat treatment, and its stability seems to be higher than the enzymes prepared from various sources (9–13). Both NADH-and NADPH-dependent GDH from turnip roots were stable at 70°C for 5 min, but when heated to 80°C the activity rapidly decreased and was totally destroyed at 85°C. Also both enzyme activities were gradually decreased by increasing the treatment time at 60°C. These results agree with our previous work with radish and corn GDH (4).

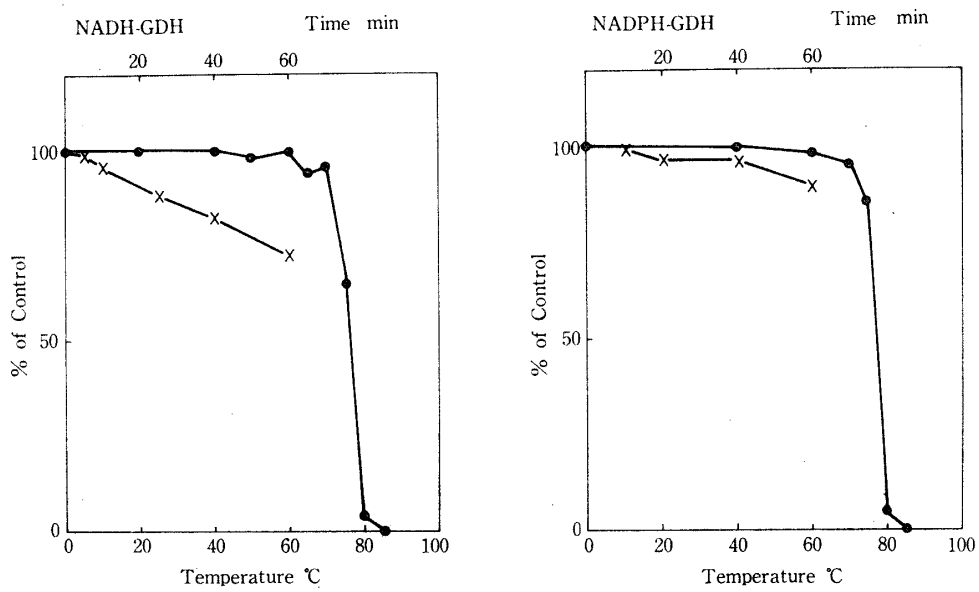

FIG. 3. Thermal inactivation curve of GDH.
(●) Changes of activities at various temperatures
(×) Changes of activities by the length of treatment time at 60°C

Inactivation by pH Changes

The activities of enzyme solutions dialyzed overnight against the buffers of various pHs are shown in Fig. 4. NADH-dependent GDH from turnip roots was more stable to pH changes than that of beef liver. It was stable at pH 4.5 without losing activity while beef liver GDH activity decreased about 85%.

Molecular Weight

Molecular weight determination of turnip GDH was carried out using 5 to 15% sucrose density gradient centrifugation at 35,000 rpm for 6 hours. Standard proteins such as bovine serum alubumin (MW: 98,000), yeast alcohol dehydrogenase (150,000), catalase (247,000), urease (473,000) and beef liver GDH (1,000,000) were used for estimating the molecular weight of turnip GDH. The sedimentation patterns of these proteins are shown in Fig 5. According to it, the molecular

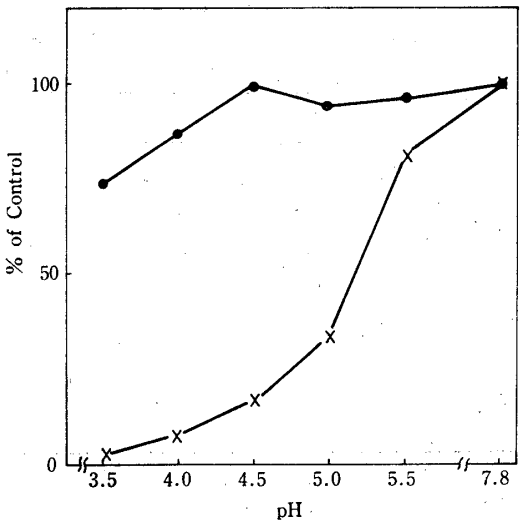

FIG. 4. Stability of NADH-GDH daialyzed against the buffers of various pHs. (●) Turnip GDH (×) Beef liver GDH

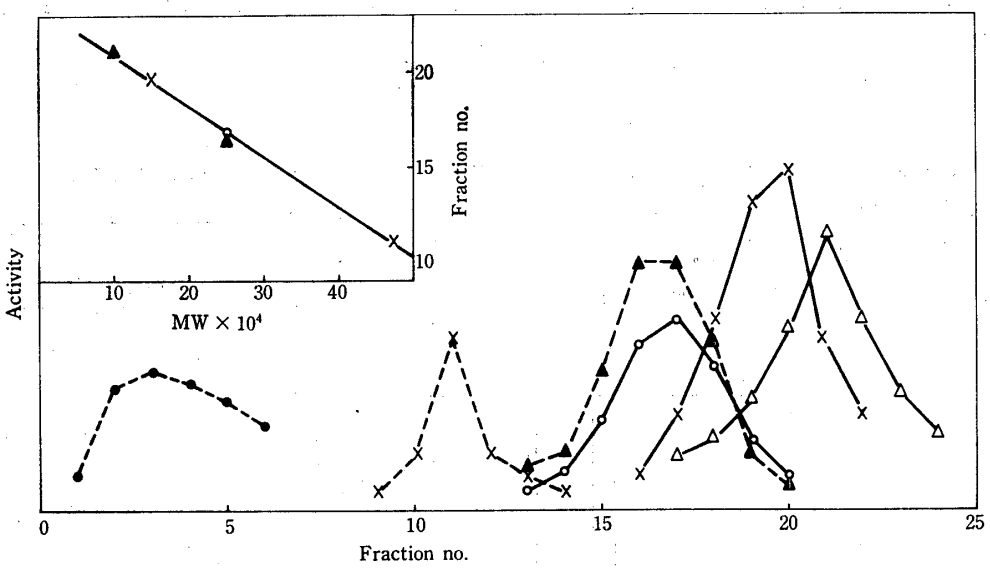

FIG. 5. Sedimentation pattern of each protein by the sucrose density centrifugation. (△—△) Albumin (×—×) Yeast ADH (○—○) Turnip GDH (▲···▲) Catalase (×···×) Urease (●···●) Beef liver GDH

weight of turnip GDH was calculated to be about 250,000. It is smaller than the values of beef liver GDH (1,000,000), chiken GDH (500,000) and neurospora NADPH-dependent GDH (330,000) (8, 10, 14). But it is larger than those of neurospora NADH-dependent GDH (200,000) and pea GDH (208,000) (13, 14).

Inhibition by Sulfhydryl Reagents

Inhibition studies were conducted with PCMB, NEM, PMA and MIA. As shown in Table 2, the sulfhydryl reagents inhibited the turnip GDH activity. Inhibitions were completely restored by the addition of GSH (1×10^{-2}M). These

TABLE 2. *Effects of Sulfhydryl Reagents on GDH Activity*

Reagent	Concentration (M)	% of control.			
		NADH-GDH	NAD-GDH	NADPH-GDH	NADP-GDH
PCMB	3×10^{-3}	81	79		
+GSH		98	95		
NEM	3×10^{-3}	98	25		
	1×10^{-3}			96	46
+GSH		100	89	100	95
PMA	3×10^{-3}	61	33		
	1×10^{-4}			59	20
+GSH		98	93	93	130
MIA	1×10^{-3}	95	97		
+GSH		100	100		

PCMB, P-chloro mercuric benzoate; NEM, N-ethyl maleiimide; PMA, Phenyl mercuric acetate; MIA, Mono iodoacetate; GSH, Reduced glutathione

results indicate that the enzymes possess sulfhydryl groups essential for their activity. Identical results have been obtained for the corn and soybean GDH (15, 16)

Effect of Cation

The effects of various cations (6.7×10^{-4}M) on turnip GDH activity are shown in Fig. 6. Ag^{+} caused the strongest inhibition upon the both NADH-and NADPH-dependent GDH activity. .

On the other hand Mg^{2+}, Mn^{2+} and Co^{2+} caused the activation of NADPH-

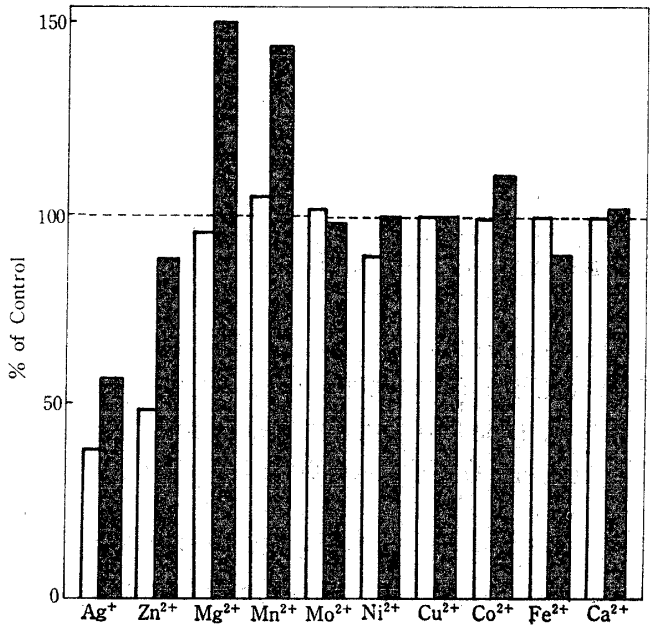

FIG. 6. Effects of various cations (6.7×10^{-4}M) on GDH activity (□) NADH-GDH (■) NADPH-GDH

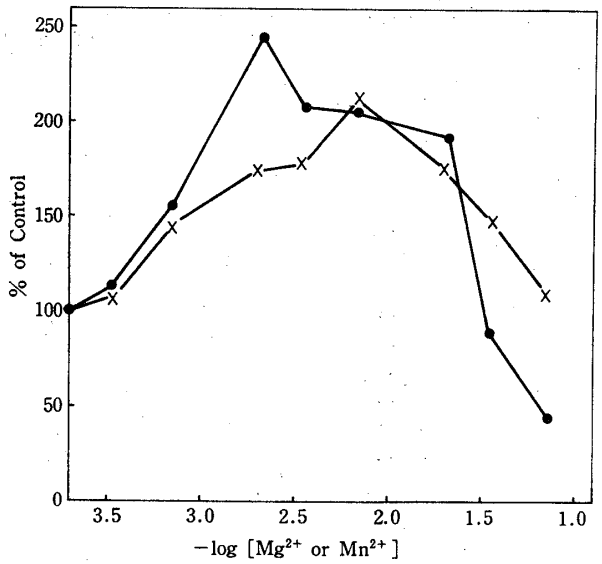

FIG. 7. Effects of Mg^{2+} and Mn^{2+} concentration on NADPH-GDH activity
(●) Mn^{2+} (×) Mg^{2+}

dependent GDH activity. To obtain more detailed results on the effects of Mg^{2+} and Mn^{2+}, the NADPH-dependent GDH activity was measured and plotted against increasing concentration of these two cations (Fig 7). The activation of its activity by Mg^{2+} or Mn^{2+} was observed at the concentrations from 6.7×10^{-2} to 6.7×10^{-4}M.

Inhibition by Chelating Agents

Table 3 shows the effects of several metal-binding inhibitors on the activities of GDH when added to the reaction mixture. Many chelating agents inhibit the activity of turnip GDH. EDTA had greater inhibitory effects upon NADH- dependent GDH than upon NAD-and NADPH-dependent GDH. To obtain more detailed results on the effect of EDTA, initial reaction velocities were measured and plotted against increasing concentration of EDTA (Fig 8). The NADH-dependent GDH activity was decreased by approximately 85% at the intermediate concentrations of EDTA, but then increased again at higher concentrations. The same tendency, though in a less degree, was also observed with NAD-dependent GDH. However the NADPH-dependent GDH activity was only decreased by about 5% at the intermediate concentrations of EDTA, and its higher concentration gave no restoration of activity. Similar results were reported for the pea and soybean GDH (13, 16, 17), but the mode of action is unknown.

The inhibition of turnip GDH activity with EDTA were restored by the addition of divallent cations such as Zn^{2+}, Cu^{2+} and Mg^{2+}.

These results indicate that GDH in turnip roots is a metallo-enzyme as recognized in other organisms (11, 13, 16, 18, 19).

TABLE 3. *Effects of Metal-binding Inhibitors on GDH Activity*

Inhibitor	Concentration (M)	% of control		
		NADH-GDH	NAD-GDH	NADPH-GDH
EDTA	1×10^{-4}	19	86	
	1.7×10^{-2}			57
Citrate	1×10^{-1}	83	100	
NTA	1×10^{-2}	24	71	91
NaN_3	1×10^{-1}	68	56	
	3×10^{-1}			10
KCN	2×10^{-3}	94	100	
TU	1×10^{-1}	83	60	
	3×10^{-1}			29
8·HQ	2×10^{-3}			65
	4×10^{-3}	48	63	
Na_2S	3×10^{-2}	94	65	
SDDC	2×10^{-2}			76
	4×10^{-2}	74	45	
OP	1×10^{-2}	67	75	60

EDTA, Disodium ethylene diamine tetraacetate; NTA, Nitriro triacetate; TU, Thiourea; 8HQ, 8-hydroxy quinoline; SDDC, Sodium diethyl dithio carbamate; OP, o-phenanthroline

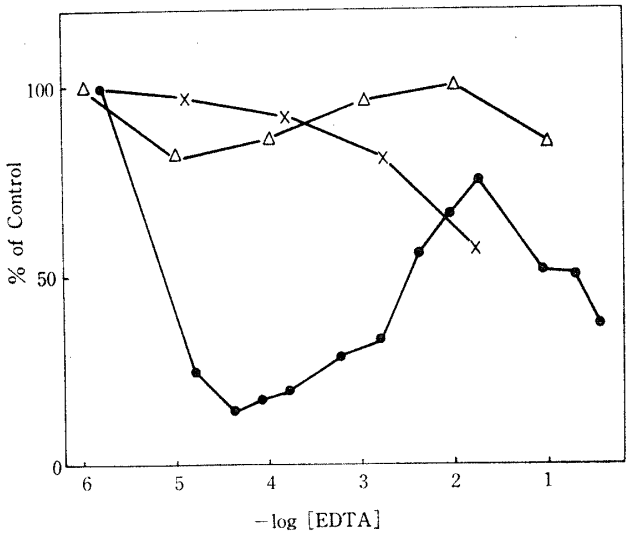

FIG. 8. Effect of EDTA concentration on GDH activity
(●) NADH-GDH (△) NAD-GDH (×) NADPH-GDH

Michaelis Constants

The Michaelis constants (Km) obtained by statistical calculation are shown in Table 4. Km values of turnip GDH are close to those of corn and soybean GDH (15, 18). It is noteworthy that Km values for ammonia are much greater than those for other substrates.

TABLE 4. *Michaelis Constants for GDH.*

Substrate	Km (M)			
	NADH-GDH	NAD-GDH	NADPH-GDH	NADP-GDH
NADH	3.7×10^{-5}			
NAD		1.7×10^{-4}		
NADPH			3.1×10^{-4}	
NADP				5.2×10^{-4}
L-glutamate		9.2×10^{-3}		3.9×10^{-3}
α-keto glutarate	4.0×10^{-3}		2.2×10^{-3}	
NH_4^+	3.5×10^{-2}		1.4×10^{-2}	

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References

1) Sims, A.P., Folkes, B.F., and Bussey, A.H., "*Recent aspects of nitrogen metabolism in plants.*" Edted by Hewitt, E.J. and Cutting, C.V. Academic Press, London. p. 91 (1968)
2) Saigusa, M., Ohira, K., and Fujiwara, A., *J. Sci. Soil and Mannure, Japan* **41** 461 (1970)
3) Saigusa, M., Ohira, K., and Fujiwara, A., *J. Sci. Soil and Mannure, Japan* **43** 371 (1972)
4) Saigusa, M., Ohira, K., and Fujiwara, A., *Soil Sci. and Plant Nutr.*, **19** 125 (1973)
5) Saigusa, M., Ohira, K., and Fujiwara, A., *J. Sci. Soil and Mannure, Japan* **44** 18 (1973)
6) Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J., *J. Biol. Chem.*, **193** 265 (1951)
7) Colowick, S.P., "*Methods in enzymology*". Edited by Colowick, S.P., and Kaplan. N.O., Academic Press, New York Vol I p. 97 (1955)
8) Olson, J.A., and Anfinsen, C.B., *J. Biol. Chem.* **167** 67 (1952)
9) Inagaki, M., *J. Biochem.*, **46** 1001 (1959)
10) Struck, J., and Sizer, I.W., *Arch. Biochem. Biophys.* **86** 260 (1960)
11) Sanwal, B.D., *Biochim, Biophys. Acta*, **93** 377 (1961)
12) Krämer, J., *Arch. Mikroviol.*, **71** 226 (1970)
13) Pahlich, E., and Joy, K.W., *Canadian J. Biochem.*: **49** 127 (1971)
14) Stachow, C.S., and Sanwal, B.D., *Biochim. Biophys. Acta.* **139** 294 (1967)
15) Bulen, W.A., *Archi. Biochem. Byophys.* **62** 173 (1956)
16) Yamasaki, K., and Suzuki, Y., *Phytochem.* **8** 963 (1969)
17) King, J., and Yung-Fun Wu, W., *Phytochem.* **10** 915 (1971)
18) Adelstein, S.J., and Vallee, B.L., *J. Biol. chem.* **233** 589 (1958) *ibid.* **234** 824 (1959)
19) Nicholas, D.J.D., and Mabey, G.L., *J. Gen. Microbiol.* **22** 184 (1960)