Plant Physiol. (1974) 53, 440–444

Nicotinamide Adenine Dinucleotide-dependent Proline Dehydrogenase in *Chlorella*

Received for publication September 24, 1973 and in revised form November 5, 1973

ALAN D. MCNAMER[1] AND CECIL R. STEWART
Department of Botany and Plant Pathology, Iowa State University, Ames, Iowa 50010

ABSTRACT

An NAD-linked dehydrogenase from *Chlorella pyrenoidosa* Chick catalyzing the conversion of L-proline to Δ^1-pyrroline-5-carboxylic acid was partially purified. Δ^1-Pyrroline-5-carboxylic acid was identified as the product by co-chromatography of it and its *o*-aminobenzaldehyde derivative with authentic compounds. The enzyme is NAD and L-proline specific and is not an oxidase; NADP is not inhibitory. The Michaelis constant for NAD is 0.08 mM and for proline is 0.73 mM.

Plants catabolize proline to glutamate (1, 5, 14, 17), and recently it has been shown that extensive catabolism of proline can occur in leaves low in carbohydrate and high in proline (17) and in *Chlorella* (14). Labeled Δ^1-pyrroline-5-carboxylic acid has been recovered from plant extracts after exposure of the tissue to ^{14}C-proline (5). In animals and microorganisms, the first enzyme in the proline to glutamate conversion is proline oxidase (2, 6, 7, 9). This enzyme requires molecular oxygen, is particulate, is inhibited by cyanide, and cytochrome *c* will serve as the electron acceptor. The product of the reaction is P5C.[2] A similar enzyme has not been isolated from plant tissue. Recently, a proline dehydrogenase has been isolated from peanut (13) and there are preliminary reports that proline dehydrogenase can be isolated from wheat germ (12) and pumpkin cotyledons (16).

This paper reports the presence of a proline dehydrogenase in extracts from *Chlorella* cells. It has been reported that proline dehydrogenase from peanut (13) does not produce P5C; however, we present evidence that P5C is the product of proline dehydrogenase from *Chlorella pyrenoidosa*. Molecular oxygen is not required for the production of P5C.

MATERIALS AND METHODS

Preparation of Cellular Material and Enzyme Extracts. Cells of *Chlorella pyrenoidosa* Chick were grown as described previously (14). Stationary phase cells were harvested at 7 days. After washing twice, the cells were resuspended in 0.1 time the original volume of 50 mM phosphate buffer (pH 7.2) containing 1 mM Na_2EDTA and 1 mM dithiothreitol. The material was passed twice through a French pressure cell at 20,000 p.s.i. Subsequently, the extracts were kept chilled on ice or the operations were done in a coldroom. After centrifugation at 1000*g* for 10 min, solid $(NH_4)_2SO_4$ was added to the supernatant at the rate of 176 g/l. After centrifugation at 10,000*g* for 10 min, the new supernatant was treated with 273 g of $(NH_4)_2SO_4$/1. After centrifugation, the pellet was dissolved in the homogenizing buffer.

Some purification was obtained by passing the extract through a DEAE-cellulose column (2.1 × 30 cm). A linear gradient of 100 ml of 0.05 M phosphate (pH 7.2) to 100 ml of 0.2 M phosphate (pH 7.2) was used to elute the enzyme. The enzyme solution was dialyzed against a saturated solution of $(NH_4)_2SO_4$ (4 C) for 12 hr. Storage was either in saturated $(NH_4)_2SO_4$ or by freezing after removal of the $(NH_4)_2SO_4$.

Assay for Proline Dehydrogenase. A typical reaction solution contained initial concentrations of 0.17 M Na-carbonate buffer (pH 10.2), 0.48 to 1.9 mM NAD, 6.7 mM proline, and 0.2 ml of enzyme extract, in a final volume of 3.0 ml. The reaction was followed spectrophotometrically at 340 nm. Enzyme activity was expressed in milliinternational units, where 1 m.i.u. is defined as 1 nmole of product formed per min. The results reported were for the initial reaction rates.

Product Identification and Stoichiometry. The ^{14}C product of proline dehydrogenase was produced and chromatographed by the method of Mazelis and Fowden (13) and in two additional solvents. The reaction mixture contained 40 mM Na-carbonate, pH 10.5, 8 mM NAD, 2 mM L-proline-U-^{14}C (specific radioactivity 0.5 μc/μmole), and 0.033 ml of enzyme extract in a final volume of 0.5 ml. After incubation at room temperature for 1 hr, 95% ethanol was added to bring the final volume to 1 ml and to terminate enzymatic reactions. After chilling for a few minutes on ice, the mixtures were centrifuged at 2000*g* for 10 min. One-fourth ml of the supernatant fraction was placed on chromatography paper (Whatman No. 3). Unlabeled proline and synthetic P5C were added to the spot for better later visualization and to prevent the complete breakdown of ^{14}C-P5C during chromatography. After chromatography, the chromatograms were cut into 1 cm and larger segments of uniform length and were eluted in counting vials. Counting procedures were those of Stewart (17), using a toluene base solvent.

The ^{14}C-complex of the product after reaction with oAB was produced for further identification. The reaction mixture contained 0.02 M Na-carbonate, pH 10.5, 0.05 mM ^{14}C-proline (specific radioactivity 1.5 μc/μmole), 4 mM NAD, and 0.5 ml of enzyme extract in a total volume of 3.0 ml. After incubation for 0.5 hr the mixture was chilled, and the pH was adjusted

[1] Present address: Department of Biochemistry, College of Medicine, University of Iowa, Iowa City, Iowa 52240.

[2] Abbreviations: P5C: Δ^1-pyrroline-5-carboxylate; GSA: glutamate-γ-semialdehyde; oAB: *o*-aminobenzaldehyde; i.u.: international unit of enzyme activity; P2C: Δ^1-pyrroline-2-carboxylate; P5C-oAB: yellow complex produced by reaction of P5C with oAB; P2C-oAB: yellow complex produced by reaction of P2C with oAB; DEAE: diethylaminoethyl.

to 7.5. Three-tenths ml of 50 mM Na-pyruvate and 10 units of lactic dehydrogenase were added, and the mixture was allowed to warm and incubate at room temperature for 10 min which was sufficient to oxidize all the NADH. Two and one-half ml of 0.5% (w/v) oAB and 5% (w/v) trichloroacetic acid were added and mixed. The mixture was incubated 0.5 hr, centrifuged, then extracted three times with ether to remove trichloroacetic acid. A control which did not have NAD was treated the same way. This material was chromatographed on Whatman No. 3 paper using the three solvents used by Costilow and Laycock (4) to resolve the P2C-oAB and P5C-oAB complexes.

For the determination of stoichiometry, the reaction mixtures contained 0.1 M Na-carbonate buffer, pH 10.5, 8 mM NAD, 6.7 mM proline, and 0.13 ml enzyme in a final volume of 2 ml. The reaction was initiated and allowed to incubate in the spectrophotometer and A_{340} was monitored. The reference cuvette did not have NAD. At the end of the incubation, the mixtures were chilled and pH was adjusted to 7.7. Three-tenths ml 50 mM Na-pyruvate and 10 units of lactic dehydrogenase were added, and the mixture was allowed to warm and incubate 10 min at room temperature to oxidize all the NADH. This procedure prevented the interference of NADH in the oAB-complexing reaction (10). Two ml of 0.5% (w/v) oAB and 20% (w/v) trichloroacetic acid in ethanol were added and allowed to stand 0.5 hr, then centrifuged. Absorbance at 443 nm was measured, and the amount of P5C was calculated using the millimolar extinction coefficient of 2.71 (19). Anaerobic incubations were done in Thunberg tubes.

Synthetic P5C was prepared by adapting the method used for synthesizing α-aminoadipic acid-δ-semialdehyde (8). Forty mg of DL-α-amino-δ-hydroxyvaleric acid (Cyclo Chemical Co., Los Angeles) and 20 mg of CrO_3 were dissolved in 5 ml of 4 N HCl. The solution was heated in a 40 C water bath for 16 hr. The HCl was evaporated to dryness, then $Cr(OH)_3$ was precipitated by adjusting the pH to 7.0 and centrifuging. The presence of P5C was verified by paper chromatography migration, by the absorption spectrum of the P5C-oAB complex, by the bright pink color of the P5C-ninhydrin complex (19), and by use of synthetic P5C as a substrate for P5C reductase.

P2C was synthesized by the method of Burton (3). P2C was identified by paper chromatography, by the absorption spectrum of the P2C-oAB complex, and by the purple color of the P2C-ninhydrin complex (18).

RESULTS AND DISCUSSION

Preparation of Proline Dehydrogenase. Table I shows the activity, specific activity, purification, and recovery of the enzyme preparation used in these experiments. The preparative steps resulted in an 11.5-fold purification giving a final specific activity of 20.3 m.i.u./mg protein. This procedure resulted in a preparation that was free of Chl, which was not removed by other methods without loss of enzyme activity. The recovery of the enzyme was 30%, and the percentage loss was about the same at each step. The greatest purification was achieved in the first ammonium sulfate fractionation, but the Chl was removed by the DEAE-cellulose chromatography.

This enzyme activity was stable for several months if stored at −15 C in 5 mM phosphate buffer, pH 7.2, even with repeated thawing and freezing. The activity was also stable when stored in saturated ammonium sulfate at 4 C. About one-half of the activity was lost in 1 month when stored in 50 mM phosphate buffer, pH 7.2, at 4 C. The activity was unstable at pH 10.2, the pH used in most assays.

This preparation contained NADH oxidase activity, P5C dehydrogenase activity, and a very large amount of P5C reductase activity. However, these enzymes were not active at pH 10.2. The P5C reductase activity at pH 8.4 was 100 times the proline dehydrogenase activity at pH 10.2. At pH values below 10, the proline dehydrogenase activity was constant for only a very short time due to the very active enzyme in the reverse direction. Thus, proline dehydrogenase was routinely assayed at pH 10.2, even though this pH may not be the optimum for proline dehydrogenase activity. The activity of the crude extracts was very low and the enzyme was concentrated during the purification as can be seen in Table I. Due to the low activity and the poor recovery, further purification was not attempted.

Table I. *Purification of Chlorella Proline Dehydrogenase*
Assays were by the NAD reduction method.

Fraction	Total Volume	Protein[1]	Total Enzyme Activity	Specific Activity	Purification Factor	Recovery
	ml	*mg*	*m.i.u.*	*m.i.u./mg*		%
1. French press supernatant	134.0	892.5	1569	1.8	1.0	100.0
2. After solid $(NH_4)_2SO_4$ treatment and resuspension	19.1	68.7	1038	15.1	8.6	66
3. After first $(NH_4)_2SO_4$ dialysis	11.1	42.5	756	17.8	10.1	48
4. After DEAE-cellulose chromatography, second $(NH_4)_2SO_4$ dialysis, and removal of $(NH_4)_2SO_4$	9.4	23.4	478	20.3	11.5	30

[1] Determined by the method of Lowry *et al.* (11) using crystalline bovine serum albumin as the protein standard.

Product Identification and Stoichiometry. Figure 1 shows the distribution of radioactivity on chromatograms of reaction mixtures containing an active and boiled enzyme incubated with ^{14}C-proline. In Figure 1A the solvent was the same as used by Mazelis and Fowden (13). The peak of radioactivity present in the active mixture but absent in the boiled mixture was in the same position relative to proline as the product was in their work. This peak occurred at the same position on the chromatogram as synthetic P5C. P2C migrated slower than proline and no radioactivity was present in that region of the chromatogram. Mazelis and Fowden (13) concluded that P5C was not the product because it was not converted to glutamic acid by H_2O_2. Johnson and Strecker (7) reported that only a small amount of P5C is converted to glutamate when treated with H_2O_2; and that most of the P5C remains unchanged. Thus, it is likely that the product of proline dehydrogenase in these experiments and those of Mazelis and Fowden (13) was P5C.

Figure 1, B and C, shows the results of chromatography of the reaction mixture in two additional solvents. In methanol-pyridine-water (20:1:5) (Fig. 1B), the peak of radioactivity present in the active mixture but absent in the boiled mixture corresponded to P5C. P2C did not separate well from proline in this solvent. The difference between the active and boiled mixture in that region of the chromatogram is interpreted as a slight (1 cm) shift in the proline peak which seems reasonable when proline had migrated nearly 40 cm. The proline peak is similarly symmetrical in the boiled and active mixture which would not be true if labeled P2C were present. In ethylacetate-formic acid-acetic acid-water (18:3:1:4) (Fig. 1C), P5C migrated as two spots, both of which reacted with oAB and were

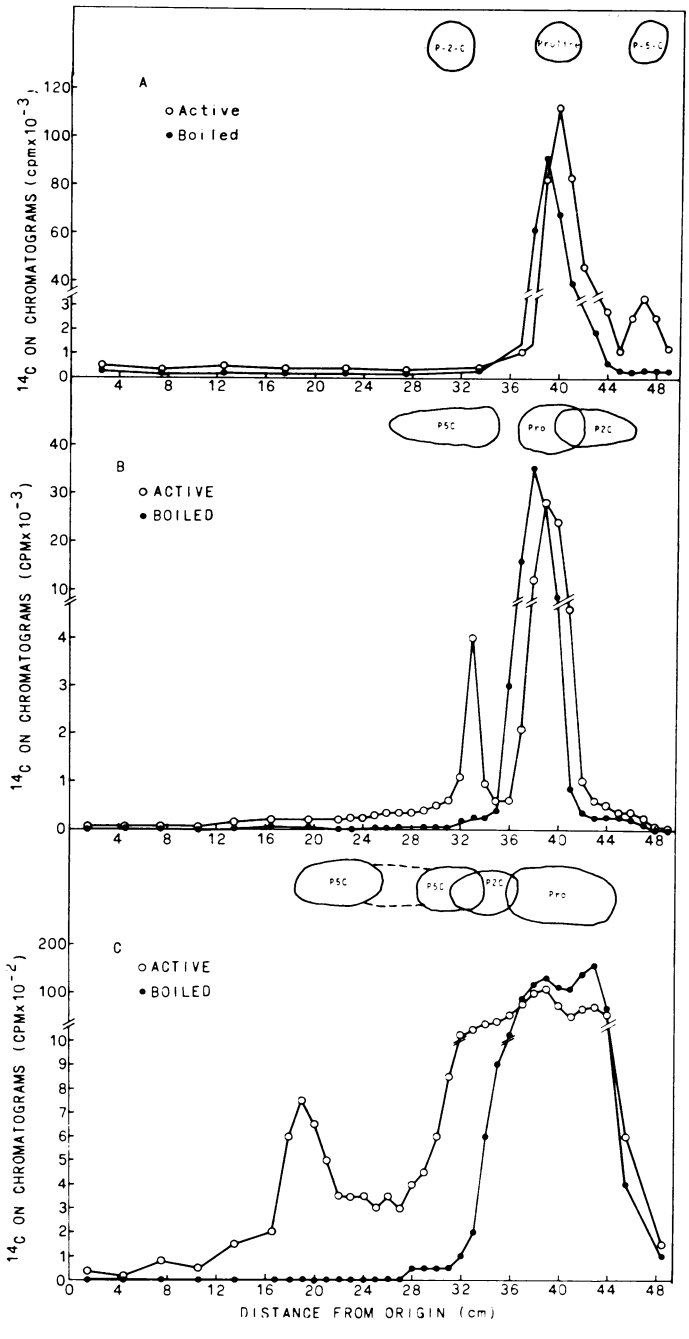

FIG. 1. Distribution of radioactivity on chromatograms after chromatography of proline dehydrogenase reaction mixtures containing an active and a boiled enzyme. Solvent systems were: A: *n*-butyl alcohol-acetic acid-water (4:1:5); B: methanol-pyridine-water (20:1:5); C: ethylacetate-formic acid-acetic acid-water (18:3:1:4). The areas of the chromatograms that were low in radioactivity were cut in larger segments and the values reported are cpm ÷ length of the segment.

pink when reacted with ninhydrin. This double spot is indicated on Figure 1C and the intense regions joined by a dotted line. The region delineated by the dotted line reacted with oAB and ninhydrin producing very faint colors. There was a difference in the radioactivity present in the active and boiled mixture which corresponded to both P5C spots. Durzan (5) observed two spots on autoradiographs after supplying ^{14}C-proline. These were claimed to be identified as P5C and GSA (5). The two spots on the chromatograms in Figure 1C could be interpreted as P5C and GSA since they are in chemical equilibrium. The most rapidly migrating spot of the two did not separate from P2C and not completely from proline. Thus, the results from chromatography in this solvent do not eliminate P2C as a product but are consistent with P5C being the product as is clear from Figure 1, A and B.

The product of the reaction reacts with oAB to produce a yellow complex. The results of attempts to chromatographically resolve the yellow P2C-oAB and P5C-oAB complexes are shown in Figure 2. The solvent systems were those used by Costilow and Laycock (4) for this purpose. In Figure 2A, the solvent was methanol-pyridine-water (20:1:5). The P5C-oAB complex separated fairly well from proline in this system but chromatographed close to P2C-oAB. However, the difference in radioactivity between the complete mixture and the one without NAD corresponded very closely to the P5C-oAB spot. Figure 2B shows the same mixtures in *n*-butyl alcohol-acetic acid-water (12:3:5). The P5C-oAB complex did not chromatograph as a compact spot in this solvent and left a "tail" as indicated in Figure 2B. However, the area on the chromatogram in which there was the greatest difference in radioactivity between the complete mixture and one without NAD corresponds to the area where the P5C-oAB complex chromatographed. There was a greater amount of ^{14}C in the complete mixture than in the one without NAD in the area of the chromatogram which corresponded to P2C-oAB but there was no peak there as would be expected since the P2C-oAB complex migrated as a compact spot. Figure 2C shows a similar distribution of ^{14}C in *n*-propyl alcohol-pyridine-water (1:1:1). The separation between proline, P5C-oAB, and P2C-oAB was not as complete as would be desirable but it is clear that the area of the chromatogram where there was the greatest difference between the complete mixture and the one without NAD corresponds to the P5C-oAB complex.

The fact that the other enzymes in the preparation which used NADH and P5C were inactive at pH 10.2 permitted the determination of the stoichiometry of the reaction. By removing the NADH after measuring the amount of it produced without destroying P5C as described under "Materials and Methods," P5C was determined by the oAB method using the extinction coefficient for the P5C-oAB published by Strecker (19). In one experiment done aerobically, 0.211 μmole of NADH and 0.208 μmole of P5C were produced. In a similar experiment done anaerobically 0.205 μmole of NADH and 0.206 μmole of P5C were produced.

Thus, based on the co-chromatography of the product with synthetic P5C in three solvents, co-chromatography of the P5C-oAB complex in three solvents, and the stoichiometric production of 1 μmole of NADH/μmole of P5C, it is clear that the product of proline dehydrogenase is P5C. The same conclusion is stated in the preliminary report of the work with proline dehydrogenase from pumpkin cotyledons (16).

Properties. The fact that the same amount of P5C was produced anaerobically as was produced aerobically (data on stoichiometry presented above), indicates that there is no proline oxidase at this pH. Azide and cyanide have no effect on the enzyme at concentrations ranging from 0.36 to 3.33 mM. Thus, the enzyme is not an oxygen-requiring enzyme as has been reported for other tissues.

Table II shows that NADP will not substitute for NAD, when proline dehydrogenase is assayed at 340 nm. Assays in which both NAD and NADP were used, exhibited the same enzyme activity, as with NAD alone. Thus, NADP is neither a substrate nor an inhibitor for this enzyme. This result is slightly different from the results of Mazelis and Fowden (13) who

found that NADP would serve to a slight extent as a substrate for the peanut enzyme. They also found NADP to be a competitive inhibitor with NAD on the peanut enzyme.

Various amino acid substrates were assayed for activity by *Chlorella* proline dehydrogenase. The results for amino acids that are normally acted on by amino acid oxidases (15) are shown in Table III. D-Proline and various L-amino acids have negligible activities. D-Proline does not inhibit the activity with

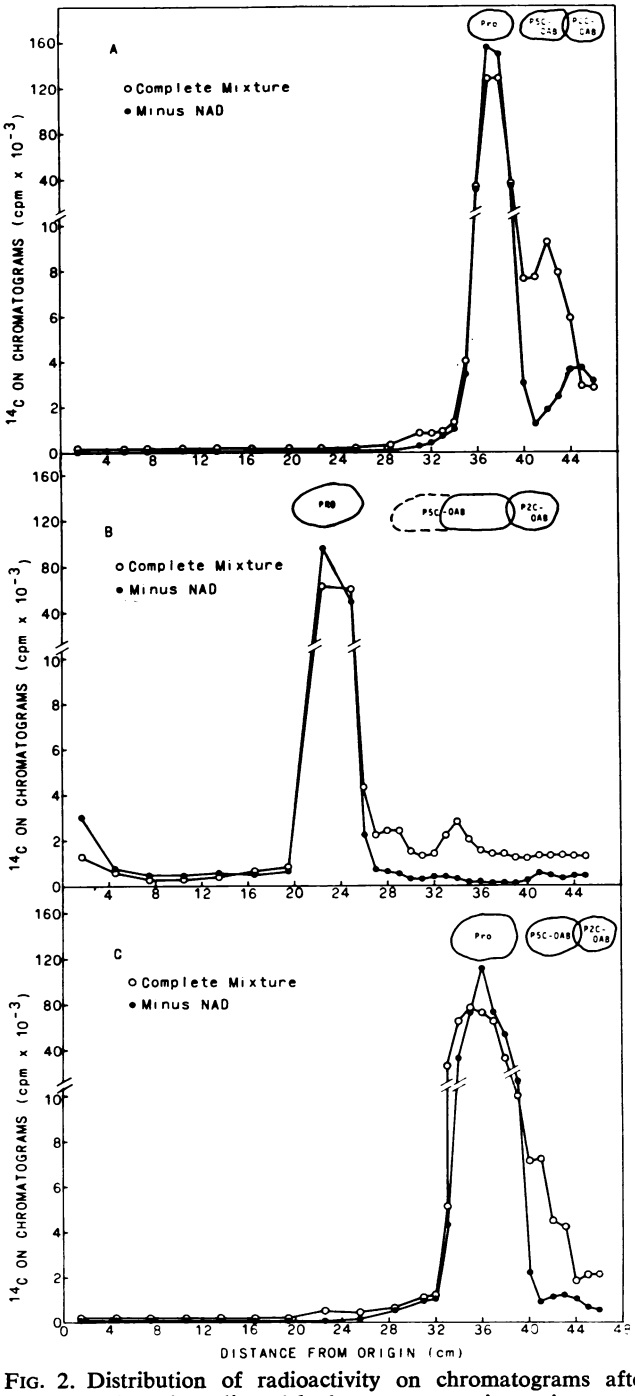

FIG. 2. Distribution of radioactivity on chromatograms after chromatography of proline dehydrogenase reaction mixtures to which oAB was added to complex the product. Solvents systems were: A: methanol-pyridine-water (20:1:5); B: *n*-butyl alcohol-acetic acid-water (12:3:5); C: *n*-propyl alcohol-pyridine-water (1:1:1). Values for the first 30 cm were determined by cutting 3-cm segments, then reporting cpm ÷ 3.

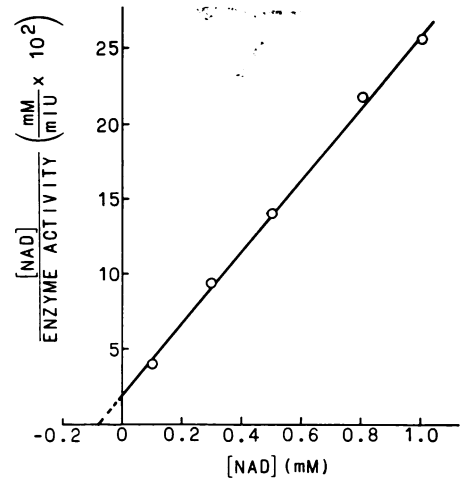

FIG. 3. Determination of the *Km* of NAD for *Chlorella* proline dehydrogenase. Proline of saturating concentration 6.7 mM was used. The activities were determined by means of the NAD reduction assay.

Table II. *Effects of NAD and NADP on Chlorella Proline Dehydrogenase Activity*

Activities were determined by means of the NAD reduction assay.

NAD	NADP	Enzyme Activity
mM		*m.i.u./ml*
0.48	0	50.1
0.48	0.44	54.4
0	0.44	0
0	0	0

Table III. *Substrate Specificity of Chlorella Proline Dehydrogenase*

All substrates had an initial reaction concentration of 6.7 mM. Activities were determined by means of the NAD reduction assay.

Substrate	Final pH	Enzyme Activity	Relative Activity
		m.i.u./ml	%
L-Proline	9.73	48.2	100
D-Proline	9.78	4.8	10
4-Hydroxy-L-proline	9.73	4.8	10
L-Glutamate	9.20	26.7	55
L-Cysteine	9.67	7.2	15
L-Serine	9.63	3.1	6
L-Alanine	9.66	3.1	6
L-Threonine	9.66	0	0

L-proline. The activity with glutamic acid cannot be interpreted due to the lack of purity of the preparation. Thus, *Chlorella* proline dehydrogenase does not appear to be either of the general amino acid oxidases, typically cataloged as EC 1.4.3.2. and EC 1.4.3.3.

The *Km* of NAD was determined for an NAD concentration range of 0 to 1.0 mM, with an initial proline concentration of 6.7 mM. The proline concentration range used for finding the *Km* of proline was 0 to 10.0 mM, with an initial NAD concentration of 0.95 mM. Figure 3 shows the determination of the

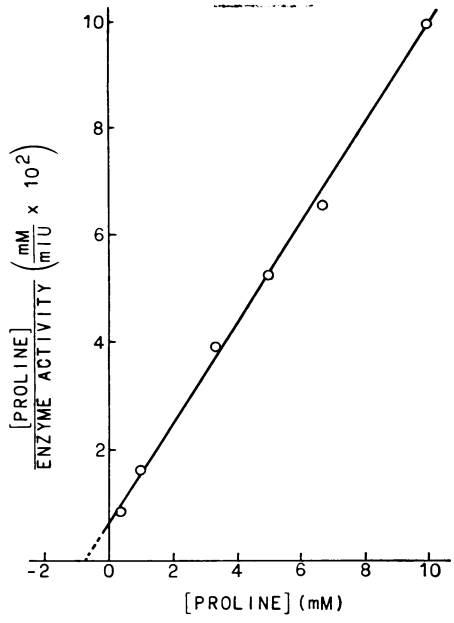

FIG. 4. Determination of the K*m* of proline for *Chlorella* proline dehydrogenase. NAD concentration was 0.95 mM. Assays were by the NAD reduction method.

K*m* of NAD, by use of the Hanes form of the Michaelis-Menten equation. The x-intercept represents the negative value of the K*m*. The K*m* has a value of 0.08 mM. Figure 4 shows the determination of the K*m* of proline. The K*m* of proline is 0.73 mM. Both K*m* values are comparable in magnitude, with those found for other plant proline dehydrogenases (12, 13).

Since the mol wt of peanut proline dehydrogenase was reported to be less than 100,000 (13) attempts were made to determine the mol wt of *Chlorella* proline dehydrogenase, using both Bio-Gel P-100 and Sephadex G-100. The enzyme activity repeatedly came off with the void volume. This is evidence that the enzyme either has a mol wt of over 100,000; or that the enzyme is bound to a complex with a combined mol wt of over 100,000. Later, Mazelis and Creveling (12) reported that the mol wt of wheat proline dehydrogenase, was in excess of 100,000.

The enzyme reactions reported in this paper were all measured at pH 10.2. No claim is made that this is the pH at which proline oxidation occurs in plant cells. The pH optimum for proline dehydrogenase is not known and repeated attempts to determine the optimum have been frustrated due to the high activity of interfering enzymes. Further, no evidence is available that this enzyme is responsible for proline oxidation in plant cells. However, this is the only enzyme that has been isolated from plants that catalyzes the oxidation of proline.

LITERATURE CITED

1. BARNARD, R. A. AND A. OAKS. 1970. Metabolism of proline in maize root tips. Can. J. Bot. 48: 1155–1158.
2. BROSEMER, R. W. AND P. A. VEERABHADRAPPA. 1965. Pathway of proline oxidation in insect flight muscle. Biochim. Biophys. Acta 110: 102–112.
3. BURTON, K. 1955. D-Amino acid oxidase from kidney. Methods Enzymol. 2: 199–204.
4. COSTILOW, R. N. AND L. LAYCOCK. 1969. Reactions involved in the conversion of ornithine to proline in *Clostridia*. J. Bacteriol. 100: 662–667.
5. DURZAN, D. J. 1973. Nitrogen metabolism of *Picea glauca*. V. Metabolism of uniformly labeled ^{14}C-L-proline and ^{14}C-L-glutamine by dormant buds in late fall. Can. J. Bot. 51: 359–369.
6. FRANK, L. AND B. RANHAND. 1964. Proline metabolism in *Escherichia coli*. III. The proline catabolic pathway. Arch. Biochem. Biophys. 107: 325–331.
7. JOHNSON, A. B. AND H. J. STRECKER. 1962. The interconversion of glutamic acid and proline. IV. The oxidation of proline by rat liver mitochondria. J. Biol. Chem. 237: 1876–1882.
8. JONES, E. E. AND H. P. BROQUIST. 1965. Saccharopine, an intermediate of the aminoadipic acid pathway of lysine biosynthesis J. Biol. Chem. 240: 2531–2536.
9. LANG, K. AND H. LANG. 1958. Der Prolin-oxydase-Komplex. Biochem. Z. 329: 577–598.
10. LARSON, R. L. AND H. P. BROQUIST. 1963. A color reaction between reduced pyridine nucleotides and *o*-aminobenzaldehyde of potential analytical use. Anal. Biochem. 5: 255–256.
11. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265–275.
12. MAZELIS, M. AND R. K. CREVELING. 1972. Proline dehydrogenase from wheat germ: purification and properties. Plant Physiol. 49: S-26.
13. MAZELIS, M. AND L. FOWDEN. 1971. The metabolism of proline in higher plants. II. L-Proline dehydrogenase from cotyledons of germinating peanut (*Arachis hypogea* L.) seedlings. J. Exp. Bot. 22: 137–145.
14. MCNAMER, A. D. AND C. R. STEWART. 1973. Proline uptake and utilization by *Chlorella pyrenoidosa*. Plant Physiol. 52: 561–564.
15. MEISTER, A. 1965. The Biochemistry of the Amino Acids, Vols. I and II. Academic Press, New York.
16. RENA, A. B. AND W. E. SPLITTSTOESSER. 1973. Proline dehydrogenase from pumpkin cotyledons. Plant Physiol. 51: S-54.
17. STEWART, C. R. 1972. Effects of proline and carbohydrates on the metabolism of exogenous proline by excised bean leaves in the dark. Plant Physiol. 50: 551–555.
18. STRECKER, H. J. 1960. The interconversion of glutamic acid and proline. II. The preparation and properties of Δ^1-pyrroline-5-carboxylic acid. J. Biol. Chem. 235: 2045–2050.
19. STRECKER, H. J. 1965. Purification and properties of rat liver ornithine-δ-transaminase. J. Biol. Chem. 240: 1225–1230.
20. TAGGART, J. V. AND R. B. KRAKAUR. 1949. Studies on the cyclophorase system. V. The oxidation of proline and hydroxyproline. J. Biol. Chem. 177: 641–653.