Purification and Properties of L-*erythro*-3,5-Diaminohexanoate Dehydrogenase from *Clostridium sticklandii*†

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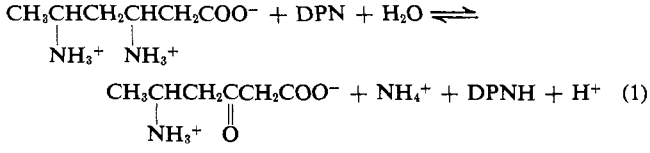
ABSTRACT: A method is described for obtaining homogeneous preparations of the dehydrogenase from *Clostridium sticklandii* that oxidatively deaminates 3,5-diaminohexanoate to 3-keto-5-aminohexanoate. The native enzyme is a dimer and has a molecular weight of about 80,000. It is specific for L-*erythro*-3,5-diaminohexanoate. Either TPN or DPN serves as coenzyme. Double-reciprocal plots of activity *vs.* pyridine nucleotide concentration are biphasic; the K_m values for TPN are lower but a higher V_{max} is observed with DPN. The apparent K_m and V_{max} values are dependent upon pH, and the concentration and type of ions that are present. Chloride, bromide, and sulfate are very inhibitory compared to arsenate and phosphate. Several divalent cations activate at pH 8.8. Dehydrogenase activity is regulated in at least two ways. TPNH and DPNH are strong product inhibitors of the oxidative deamination. ATP is a feedback inhibitor which is competitive with both 3,5-diaminohexanoate and pyridine nucleotide. These metabolic controls suggest that the dehydrogenase plays an important role in controlling the rate of lysine fermentation. The dehydrogenase from *C. sticklandii* differs markedly in physical properties and in regulatory characteristics from the 3,5-diaminohexanoate dehydrogenase of *Clostridium* strain SB_4.

Baker *et al.* (Baker, 1970; Baker and Barker, 1970; Baker *et al.*, 1972) purified and partially characterized a DPN-dependent 3,5-diaminohexanoic acid dehydrogenase from extracts of *Clostridium* strain SB_4. This enzyme catalyzes the oxidative deamination of 3,5-diaminohexanoate to 3-keto-5-aminohexanoate (eq 1). *Clostridium sticklandii* extracts also



$$CH_3CHCH_2CHCH_2COO^- + DPN + H_2O \rightleftharpoons CH_3CHCH_2CCH_2COO^- + NH_4^+ + DPNH + H^+ \quad (1)$$

contain this dehydrogenase, but its properties are remarkably different from the *Clostridium* SB_4 dehydrogenase. In this paper we report the purification and properties of the 3,5-diaminohexanoate dehydrogenase from *C. sticklandii*.

Materials and Methods

C. sticklandii was cultured and harvested as previously described by Stadtman (1966). Cells were stored at −80°.

Synthetic 2,5-diaminohexanoate, 2,4-diaminopentanoate, the lactams of DL-*erythro*- and DL-*threo*-3,5-diaminohexanoate, and 2-methylpyrrolidone-5-carboxylic acid were gifts from Dr. L. Tsai. DL-*erythro*- and D-*erythro*-3,5-diaminohexanoate were gifts from Dr. H. A. Barker. L-β-Lysine was obtained from Cyclo Chemical Corp. 1,4-Dithiothreitol and pCl-HgBzO[1] were purchased from Calbiochem, DPN(H), TPN(H), deamino-DPN, acetylpyridine-DPN, ATP, and other nucleotides from P-L Biochemicals.

Hydroxylapatite was prepared by the method of Tiselius *et al.* (1956).

Standard Assay. The dehydrogenase was assayed at 37° by following DL-*erythro*-3,5-diaminohexanoate-dependent formation of DPNH from DPN^+ at 340 nm with a Gilford Model 2000 multiple-sample absorbance recorder. The assay mixture (1 ml) contained 0.4 mM DPN, 1 mM 3,5-diaminohexanoate-2HCl, and 100 mM ethanolamine-HCl buffer (pH 9.0). Usually the reaction was started by the addition of enzyme to the otherwise complete mixture. The activity was linear with the amount of enzyme up to 0.07 unit/assay. One unit of activity is defined as the amount of enzyme which produces 1 μmol of DPNH/min.

Protein was determined according to Lowry *et al.* (1951) using bovine serum albumin as standard.

Polyacrylamide and sodium dodecyl sulfate gel electrophoreses were carried out as described before (Baker *et al.*, 1973). Protein and dehydrogenase activity stains were done as described by Baker *et al.* (1972).

Results

Purification of the Enzyme. All steps were performed at 4°. Except where specified otherwise, the standard buffer used throughout the purification consists of 20 mM potassium phosphate–3 mM NaEDTA (pH 6.8).

1. CELL-FREE EXTRACT. *C. sticklandii* cells (50 g) were suspended in 125 ml of 100 mM potassium phosphate–3 mM NaEDTA buffer (pH 7.5) and sonicated with cooling in 40-ml batches for 3 min with a Branson Sonifier (Model LS-75). The broken cell suspension was centrifuged for 25 min at 18,000*g*. The cell-free supernatant was used for the next step.

2. AMMONIUM SULFATE PRECIPITATION. Solid ammonium sulfate (32.6 g/100 ml) was added slowly to bring the supernatant to 55% saturation. After 30 min the precipitate was removed by centrifugation for 25 min at 18,000*g*, and 16.1 g of ammonium sulfate/100 ml was added to bring the supernatant

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[1] Abbreviations used are: pCl-HgBzO, *p*-chloromercuribenzoic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

TABLE I: Purification of 3,5-Diaminohexanoate Dehydrogenase from *C. sticklandii*.

Step of Purification	Vol (ml)	Protein (mg)	Act. (μmol/min)	Sp Act. (units/mg)
1. Crude extract	137	5480	854	0.156
2. 55–80% ammonium sulfate	43	1455	596	0.412
3. Sephadex G-25	70	1302	497	0.382
4. DEAE-cellulose	75	338	464	1.28
5. Sephadex G-100	22	77	352	4.57
6. Hydroxylapatite	115	34.4	324	9.4
7. DEAE-Sephadex	52	12.7	202	15.9

to 80% saturation. After 30 min the precipitate was collected by centrifugation and taken up in standard buffer. The latter fraction contained 70% of the total dehydrogenase activity.

3. SEPHADEX G-25 CHROMATOGRAPHY. The enzyme from step 2 was desalted on a column (4 × 43 cm) of Sephadex G-25. The column was equilibrated and eluted with the standard buffer.

4. DEAE-CELLULOSE CHROMATOGRAPHY. The desalted protein solution was applied to a column (4.2 × 20 cm) of DEAE-cellulose (Whatman DE-52) which had been equilibrated with the standard buffer. The protein was eluted with a 1-l. linear gradient of NaCl (0–0.3 M) in the standard buffer, and 6.6-ml fractions were collected. The dehydrogenase eluted between 0.16 and 0.18 M NaCl (fractions 95–106).

5. SEPHADEX G-100 CHROMATOGRAPHY. Fractions 95–106 from step 4 were pooled and concentrated by bringing the solution to 85% saturation (55.9 g/100 ml) with ammonium sulfate. The precipitate was collected by centrifugation and dissolved in 10 ml of standard buffer. This concentrated protein solution was applied to a column (2.5 × 100 cm) of Sephadex G-100, which had been equilibrated with 50 mM Tris-HCl–3 mM NaEDTA buffer (pH 7.5). The protein was eluted with the same pH 7.5 buffer, and 2-ml fractions were collected. Fractions 68–77, which contained 76% of the dehydrogenase activity applied to the column, were pooled.

6. HYDROXYLAPATITE CHROMATOGRAPHY. Fractions 68–77 from step 5 were applied to a hydroxylapatite column (3 × 10 cm) equilibrated with 50 mM Tris-HCl–3 mM NaEDTA buffer (pH 7.5). A stepwise elution was started with 25 mM potassium phosphate–1 mM NaEDTA buffer (pH 6.8). Fractions of 4.6 ml were collected. When dehydrogenase activity appeared in the eluate, the phosphate concentration was raised to 100 mM, which removed the dehydrogenase in a sharp peak.

7. DEAE-SEPHADEX CHROMATOGRAPHY. The dehydrogenase from step 6 was applied to a column (1.8 × 25 cm) of DEAE-Sephadex A-50 equilibrated with 0.1 M NaCl in standard buffer. Protein was eluted with a 600-ml linear gradient of NaCl (0.1–0.4 M) in the standard buffer, and 3-ml fractions were collected. Fractions 80–97 were pooled and concentrated by vacuum dialysis. The concentrated solution (12.7 mg of protein in 2.9 ml) was used to characterize the dehydrogenase.

Table I gives a summary of the purification procedure. The dehydrogenase was purified 102-fold with a recovery of 24%.

Stability. No loss of activity of the crude enzyme (0.1–20 mg of protein/ml) was observed after 1 day at 23°, 1 week at 4°, or 1 month at −20°. After purification step 7, the activity of the dehydrogenase (0.1–4.4 mg of protein/ml) did not decrease after 2 months at 4°. However, when stored

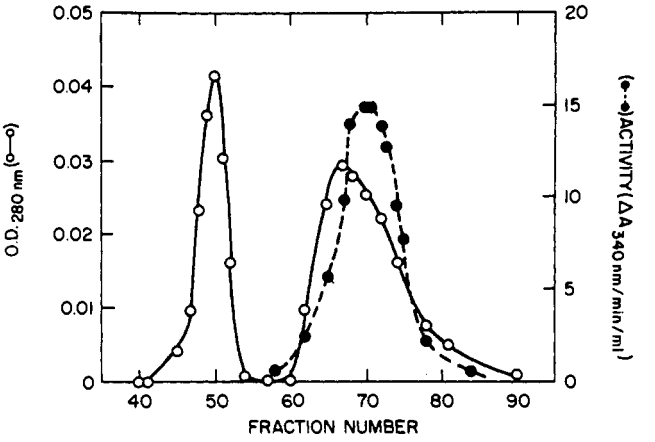

FIGURE 1: Sephadex G-100 chromatography of dehydrogenase stored for 9 weeks at −20°. Fractions of 2 ml were collected; further details are given in the text.

at −20° with some thawing and refreezing, the activity decreased 10% after 3 weeks and 60% after 9 weeks. Examination of the 60% inactivated preparation by gel filtration on Sephadex G-100 disclosed two protein peaks (Figure 1). The first peak elutes in the void volume; it contained 38% of the protein placed on the column, but less than 0.3% of the total activity. The second peak (62% of the protein) contained 99% of the dehydrogenase activity. Although the activity peak is in its normal position, it does not coincide with the second protein peak indicating that inactive protein was also present.

The high molecular weight protein fraction (peak 1 of Figure 1) was partially converted to active dehydrogenase by treatment with 2 mM dithiothreitol and 8 M urea in 20 mM potassium phosphate–3 mM NaEDTA buffer (pH 6.8) for 48 hr at 4°. The specific activity of the preparation, measured after removal of urea by passage over Sephadex G-25, was increased 18-fold. Treatment with urea or dithiothreitol alone had no effect indicating that both are required for restoration of activity. Rapid freezing of enzyme solutions rather than allowing them to freeze slowly at −20° did not prevent inactivation and polymerization of the purified dehydrogenase.

Homogeneity. After storage at −20° for 3 weeks, the homogeneity of the dehydrogenase from step 7 was determined by gel electrophoresis at pH 8.5 in 5, 7.5, and 10% polyacrylamide gels (Figure 2). At this time the preparation was 10% less active than when first isolated. In each of the three gels, the protein stain shows a major band, two minor bands, and a band at the top of the gel. The dehydrogenase activity stain shows that most of the dehydrogenase activity coincides with the major protein band and that faint activity bands coincide with one of the minor protein bands and with the protein at the top of the gel. The bands at the top of the gels are protein that polymerized as a result of freezing (see preceeding section on Stability). Excluding this polymerized material, which is absent in newly isolated dehydrogenase preparations, the enzyme appears to be at least 95% pure.

Molecular Weight. Molecular weight determination by gel filtration (Andrews, 1965) on a Sephadex G-150 column (2.5 × 45 cm) which had been standardized with proteins of known molecular weight gave a value of 82,000. Molecular weight determination by the gel electrophoretic procedure of Hedrick and Smith (1968), using the activity stain to locate the dehydrogenase, gave a value of 79,000. The monomer molecular weight was determined by gel electrophoresis in the

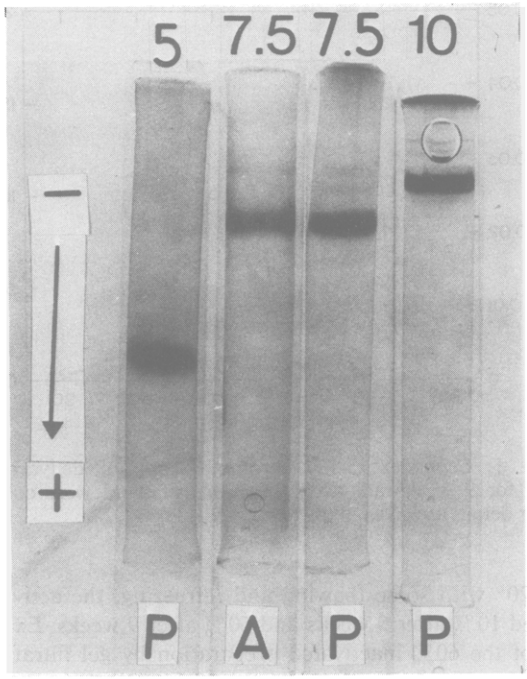

FIGURE 2: Polyacrylamide gel electrophoresis of 3,5-diaminohexanoate (DAH) dehydrogenase purified through step 7. The per cent acrylamide concentration is indicated at the top of each gel. "P" indicates that the Amido Black protein stain was used; "A" indicates the use of a specific activity stain. Protein (50 μg) was placed on each gel. The direction of protein movement is indicated by the arrow. Other details are given in the text or in the mehods.

presence of sodium dodecyl sulfate. A value of 39,300 was obtained indicating that the dehydrogenase is isolated as a dimer.

The dimer molecular weight determined by these three methods is in good agreement and is about 80,000.

Heat Stability. The effect of incubation of the enzyme at temperatures between 40 and 60° was examined. No loss of activity occurred during 30 min at 40°, but at higher temperatures, the inactivation followed first-order kinetics with $t_{1/2}$ = 56 min at 50°, $t_{1/2}$ = 32 min at 55°, and $t_{1/2}$ = 4 min at 59°.

The heat stability at 55° was also determined in the presence of DPN (0.4 mM), 3,5-diaminohexanoate (5 mM), or with both together. After 30 min, 54% of the activity remained in the absence of substrates, 24% in the presence of DPN, 75% with 3,5-diaminohexanoate, and 94% with both DPN and 3,5-diaminohexanoate. Thus, in the presence of both DPN and 3,5-diaminohexanoate, the enzyme is effectively stabilized against heat inactivation at 55°. DPN alone accelerates heat inactivation.

pH Stability. The effect of pH on the stability of the enzyme was studied by diluting the enzyme (final concentration 0.44 mg of protein/ml) tenfold into 100 mM buffer. The enzyme was inactivated instantaneously at pH 4.5 (acetate buffer) and at pH 12 (0.01 N NaOH). In the pH range 5–10.5 no loss of activity was observed after 30 min at 25°.

Kinetic Properties and Specificity

Effect of pH on Velocity. Figure 3 shows that the optimal pH for the dehydrogenase activity with either TPN or DPN as coenzyme is between pH 8.7 and 9.0. At the maximum, the activity in ethanolamine-HCl buffer is 1.16-fold higher than that observed in diethanolamine-HCl.

TABLE II: Substrate Specificity and Competitive Inhibitors.

Compound	Rel Act.	K_i (mM)
DL-*erythro*-3,5-Diaminohexanoate	1.0	
D-*erythro*-3,5-Diaminohexanoate	$<10^{-4}$	0.1[a]
2,5-Diaminohexanoate	$<10^{-4}$	0.19
2,4-Diaminopentanoate	$<10^{-4}$	0.52
L-Lysine	$<10^{-4}$	2.5
L-β-Lysine	$<10^{-4}$	2.5
D-Lysine	$<10^{-4}$	11.3
L-Ornithine	$<10^{-4}$	5.4
DL-β-Aminobutyrate	$<10^{-4}$	73.4

[a] Since DL-*erythro*-3,5-diaminohexanoate was used as the variable substrate, the concentration of D-*erythro*-3,5-diaminohexanoate also varied. The K_i was estimated from Dixon plots using corrected D-*erythro*-3,5-diaminohexanoate concentration. Substrate specificity was determined with 3.5 μg of protein in 100 mM diethanolamine-HCl (pH 8.8) in the presence of 0.4 mM DPN and 10 mM of the indicated amino acids. The K_i values were determined in reaction mixtures containing 88 mM triethanolamine-HCl (pH 7.4), 0.87 μg of protein, and 0.4 mM TPN. The concentration of 3,5-diaminohexanoate was varied in the presence of a constant amount of inhibitor. The apparent K_m for 3,5-diaminohexanoate was determined from a Lineweaver–Burk plot with each inhibitor. For competitive inhibition, K_i can be calculated from the apparent K_m which is equal to the K_m observed in the absence of inhibitor times $(1 + [I]/K_i)$, where [I] is the inhibitor concentration.

Substrates and Inhibitors. Table II shows the ability of compounds structurally related to 3,5-diaminohexanoate to act as substrates or as competitive inhibitors. The enzyme is specific for DL-*erythro*-3,5-diaminohexanoate. The L isomer is the actual substrate for the enzyme; however, it is difficult to obtain whereas the DL pair is readily synthesized. D-*erythro*-3,5-Diaminohexanoate, 2,5-diaminohexanoic acid, and 2,4-diaminopentanoic acid are the most effective inhibitors. L-Lysine, L-β-lysine, L-ornithine, and D-lysine are less effective, and β-aminobutyrate is a poor inhibitor. Although not shown in the table, the lactams of DL-*erythro*-3,5-diaminohexanoate and DL-*threo*-3,5-diaminohexanoate, and 2-methylpyrrolidone-5-carboxylic acid had no effect on activity.

Coenzyme Specificity. The effectiveness of several pyridine nucleotides to act as coenzyme at pH 8.8 is shown in Table III. Two apparent K_m and V_{max} values are shown for TPN because the Lineweaver–Burk plot is biphasic as shown in Figure 4A. A similar plot with DPN (Figure 4B) gives a straight line with a slight downward falloff at high DPN concentrations. By using even higher DPN concentrations, minimum values of the second K_m and V_{max} for DPN were estimated (Table III). Straight lines were obtained with deamino-DPN and acetylpyridine-DPN.

Although DPN has a higher V_{max}, TPN is a more effective substrate because its K_m values are much smaller. Deamino-DPN is a poor substrate because of its high K_m; acetylpyridine-DPN is a poor substrate because of its low V_{max}.

Effect of pH on K_m and V_{max} Values. Table IV shows the apparent K_m and V_{max} values obtained at pH 7.4. The values are lower than those obtained at pH 8.8 (Table III), indicating that they are pH dependent.

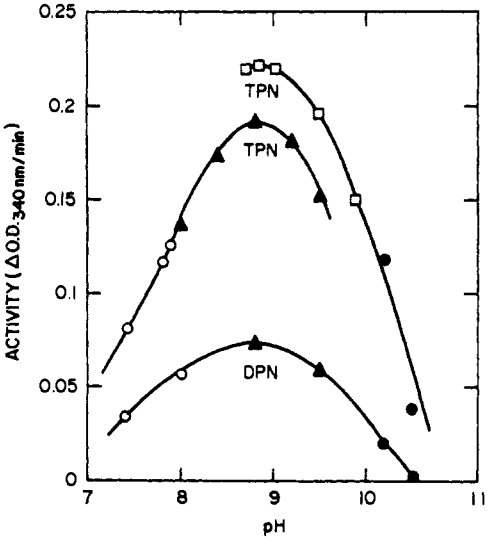

FIGURE 3: The effect of pH on dehydrogenase activity. Assays were performed by the standard procedure, except 100 mM triethanolamine-HCl (○), diethanolamine-HCl (▲), ethanolamine-HCl (□), or sodium carbonate (●) was used at the indicated pH values with TPN or DPN. Each point was obtained with 0.85 μg of enzyme.

The apparent K_m and V_{max} values for 3,5-diaminohexanoate are also pH dependent. As shown in Table IV, at pH 7.4 the K_m is 0.08 mM with TPN and 0.16 mM with DPN; V_{max} is 19.4 units/mg with TPN and 11.1 units/mg with DPN. In 100 mM diethanolamine-HCl at pH 8.8, the K_m for 3,5-diaminohexanoate is 2.0 mM with both coenzymes and V_{max} is 106 units/mg with TPN and 57.1 units/mg with DPN. The results differ depending on whether DPN or TPN is the fixed substrate because the concentration used (0.4 mM) was not saturating, particularly for DPN.

Effect of Ions on K_m and V_{max} Values of Pyridine Nucleotides. The K_m and V_{max} values of the pyridine nucleotides are also dependent upon the nature and concentration of the ions which are present. Specific effects differ slightly depending on the pH and the pyridine nucleotide used. For example, when the dehydrogenase was assayed in 44 mM triethanolamine-HCl buffer (pH 7.4), the apparent K_m for DPN is 0.1 mM and V_{max} is 6.12 units/mg. Both values are severalfold lower than those shown in Table IV which were obtained in 88 mM triethanolamine-HCl.

The presence of neutral salts also affect the K_m and V_{max} values. Addition of 80 mM KCl to 44 mM triethanolamine-HCl (pH 7.4) causes the K_m to increase to 0.4 mM and V_{max} to 24.6 units/mg. Similar results are observed with NaCl, NH_4Cl, KBr, or K_2SO_4.

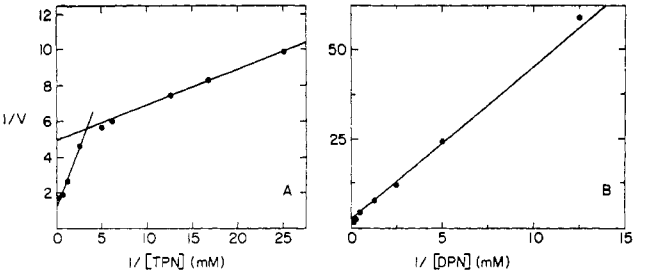

FIGURE 4: Double-reciprocal plots of activity *vs.* TPN concentration (A) or DPN concentration (B). Activity (ΔOD/min) was determined in reaction mixtures (1 ml) containing 88 mM diethanolamine-HCl (pH 8.8), 1.0 mM DAH, and 0.87 μg of protein. The concentration of DPN or TPN was varied as indicated.

The nature of these ionic effects has not been well studied at pH 7.4 because Lineweaver–Burk plots have to be done at every salt concentration to separate the K_m and V_{max} effects. Generally, V_{max} increases with salt or buffer concentration until a maximum value is obtained. K_m continues to increase with ion concentration, and a severe inhibition is observed at high ion concentrations. At lower ion concentrations, the activity of the dehydrogenase can increase, stay constant or decrease as the ion concentration increases depending upon whether the V_{max} or K_m effect is more important under the particular assay conditions employed.

Ions have simpler effects on the enzyme at higher pH values. For example, at pH 9.0 in 20 and 100 mM ethanolamine-HCl buffer, double-reciprocal plots of activity *vs.* DPN concentration intersect on the ordinate (the apparent K_m values are 1.0 and 3.3 mM, respectively) indicating that ions act as competitive inhibitors to pyridine nucleotide; therefore, only the K_m effect is important. The order of the inhibition is shown in Figure 5 where $1/V$ has been plotted as a function of the ethanolamine buffer concentration, [EA]. When the data are replotted as $(1/V - 1/V_{max})/[EA]$, a straight line is obtained, proving that the original plot is a parabola. This indicates that more than one ion combines with the enzyme and the inhibition is parabolic competitive. The parabola shown in Figure 5 is actually one of a family of parabolas which inter-

TABLE III: Effect of Pyridine Nucleotides on Activity at pH 8.8.

Pyridine Nucleotide	K_m[a] (mM)	V_{max}[a] (units/mg)
TPN	0.04	38.5
	1.25	186
DPN	1.06	65
	>10[b]	>206[b]
Deamino-DPN	22	51.5
Acetylpyridine-DPN	3.3	20.8

[a] These values were taken from Lineweaver–Burk plots. It is assumed that the biphasic TPN and DPN saturation functions are not due to negative cooperativity. Assays were done as described in Figure 4 except the indicated pyridine nucleotide was used. [b] These are estimates of the minimum possible values.

TABLE IV: K_m and V_{max} Values in Triethanolamine-HCl Buffer (pH 7.4).

Pyridine Nucleotide	K_m (mM)	V_{max} (units/mg)
DPN	0.3	15.9
	>5.0[a]	>39.4[a]
TPN	0.03	17.1
	0.16	22.5
3,5-Diaminohexanoate	0.08[b]	19.4[b]
	0.16[c]	11.1[c]

[a] These are estimates of the minimum possible values. [b] Determined in the presence of 0.4 mM TPN. [c] Determined in the presence of 0.4 mM DPN. This experiment was done as described in Table III except the buffer was 88 mM triethanolamine-HCl (pH 7.4). When 3,5-diaminohexanoate was varied, the DPN or TPN concentration was held constant at 0.4 mM.

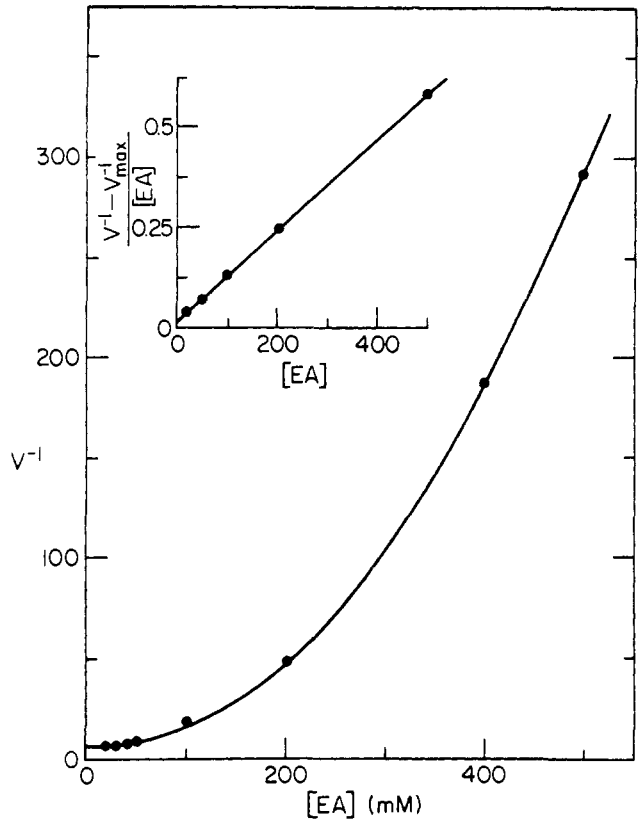

FIGURE 5: Reciprocal activity (V^{-1}) *vs.* ethanolamine-HCl buffer concentration [(EA)] at pH 9.1. Activity (ΔOD/min) was determined with 0.4 mM DPN, 1.0 mM DAH, and 0.50 μg of enzyme in the indicated ethanolamine-HCl concentration. For the inset plot of $V^{-1} - V_{max}^{-1}/[EA]$ *vs.* [EA], V_{max}^{-1} is the intercept on the V^{-1} axis of the larger graph.

sect to the left of the $1/V$ axis. Another enzyme in which salt acts as a parabolic inhibitor is β-hydroxybutyrate dehydrogenase (Nielsen *et al.*, 1973).

Additions of neutral salts such as KCl, NaCl, NH_4Cl, KBr, or K_2SO_4 to the assay solution at pH 9.0 also cause a parabolic inhibition. The inhibitory agent in ethanolamine-HCl buffer is mainly the chloride ion because sodium ethanolamine arsenate buffer (pH 9.0) is a poor inhibitor compared to ethanolamine-HCl.

The effect of ions on the K_m and V_{max} values of TPN is more difficult to study because of the biphasic kinetics. However, the effects are similar to those described for DPN except V_{max} increases with increasing salt or buffer concentration at pH 9.0 as well as at pH 7.4. The K_m values increase with increasing salt concentration, and a severe inhibition is observed at high concentrations.

Since sodium ethanolamine arsenate buffer is not inhibitory compared to ethanolamine-HCl buffer, the K_m and V_{max} values of DPN, TPN, and 3,5-diaminohexanoate were determined at pH 7.4 and 9.1 in sodium arsenate buffer. The results are shown in Table V. The V_{max} values for DPN are over twice as high as those found in triethanolamine- and diethanolamine-HCl buffers at comparable pH values. The K_m value is about the same at pH 7.4, but is lower in arsenate at pH 9.1 than in diethanolamine-HCl at pH 8.8. The second K_m value for TPN is much lower in the arsenate buffers and three of the four V_{max} values are higher. At pH 7.4, the same results for DPN are obtained in potassium or sodium phosphate.

The apparent K_m and V_{max} values are dependent upon the

TABLE V: K_m and V_{max} Values in Arsenate Buffer.[a]

Pyridine Nucleotide	K_m (mM)		V_{max} (units/mg)	
	pH 7.4	pH 9.1	pH 7.4	pH 9.1
DPN	0.48	0.67	39	138
TPN	0.03	0.05	18.6	101
	0.07	0.27	27.6	150

[a] This experiment was done as described in Table III except 88 mM sodium arsenate buffer was used at the indicated pH.

arsenate concentration. For example, at pH 9.1, in 20 mM arsenate, DPN has a K_m value of 0.3 mM and V_{max} of 72 units/mg; TPN has V_{max} values of 90 and 103 units per mg and K_m values of 0.03 and 0.09 mM.

Addition of any of the neutral salts previously mentioned to the arsenate buffer causes a parabolic inhibition. A direct comparison of arsenate and chloride ion shows that arsenate is not as inhibitory even though it has a higher ionic strength. For example, at pH 9.1, sodium arsenate has an ionic strength 4.5-fold higher than NaCl. Therefore, the ionic effects are not a general ionic strength effect, but are caused by the action of specific ions on the enzyme. Arsenate (and phosphate) clearly interacts with the enzyme differently from chloride and bromide. Sulfate, which is a large ion like arsenate, is as inhibitory as chloride (at equivalent ionic strength) with DPN as coenzyme, but it is much less effective than chloride with TPN as coenzyme.

Effect of Metal Ions on Activity. Table VI shows the effect of metal ions on the enzymic reaction. At pH 7.8 all cations tested are inhibitory except Fe^{3+} which has no effect. At pH 8.9, Mn^{2+}, Ca^{2+}, Zn^{2+}, Co^{2+}, and Mg^{2+} increase the enzymic activity, but Cu^{2+}, Fe^{2+}, and Fe^{3+} inhibit slightly. Mn^{2+} gives the highest increase in activity (232% at 0.5 mM); concentrations of 0.01, 0.05, 0.1, 0.75, and 1.0 mM increase the enzyme activity by 110, 156, 188, 258, and 263%, respectively. The activity was estimated to be maximal at 0.75 mM Mn^{2+}; however, the optimal concentrations could not be determined accurately because turbidity, probably due to formation of $Mn(OH)_2$, was observed at concentrations of 1 mM Mn^{2+} and above.

The results shown in Table VI were obtained with TPN as the cofactor. Similar results were observed with DPN.

The effect of Mn^{2+} on the K_m and V_{max} values of the enzyme was investigated at pH 8.9. In the presence of 0.5 mM $MnCl_2$, the K_m for 3,5-diaminohexanoate decreased from 2.0 to 0.3 mM and V_{max} increased from 57.1 to 70 units per mg. The K_m and V_{max} values for DPN were also much lower in the presence of Mn^{2+}. The double-reciprocal plot of activity *vs.* DPN concentration had a downward curvature. The range of DPN concentration used was not broad enough to determine the linear portions of the curve very accurately, but approximate K_m values were estimated to be 0.03 and 0.14 mM with corresponding V_{max} values of 39 and 64 units per mg.

Inhibition by pCl-HgBzO. The dehydrogenase is inhibited by the organic mercurial, pCl-HgBzO. Preincubation of the enzyme (4.2 μg of protein/ml) for 10 min in the standard assay mixture (without 3,5-diaminohexanoate) in the presence of pCl-HgBzO (≥1 μM) resulted in complete inhibition. At a pCl-HgBzO concentration of 0.3 μM, 36% inhibition was observed. When the enzyme was exposed to pCl-HgBzO and substrate simultaneously, no inhibition was observed with 1

TABLE VI: Effect of Metal Ions on Dehydrogenase Activity.[a]

Cation (0.5 mM)	Act. (%) pH 7.8	pH 8.9
None	100	100
Mn^{2+}	48	232
Ca^{2+}	78	208
Zn^{2+}	51	157
Co^{2+}	29	150
Mg^{2+}	37	136
Fe^{2+}	64	91
Fe^{3+}	100	92
Cu^{2+}	65	70

[a] Reaction mixtures (1 ml) contained 100 mM potassium–Hepes buffer (pH 7.8) or 100 mM ethanolamine-HCl buffer (pH 8.9), 0.4 mM TPN, 1 mM 3,5-diaminohexanoate, 0.87 μg of enzyme, and the indicated metal ion salt. Mn^{2+}, Ca^{2+}, Co^{2+}, and Fe^{3+} were chloride salts; the other metal ions were sulfate salts.

μM pCl-HgBzO but 0.1 mM pCl-HgBzO caused instantaneous inactivation.

Effect of Temperature on Activity, The effect of temperature on the rate of the dehydrogenase catalyzed reaction in 100 mM ethanolamine-HCl buffer (pH 8.9) is shown in Figure 6. With TPN the activity increases up to 52° and then rapidly falls off, probably due to heat inactivation of the protein. With DPN, the activity of the enzyme increases up to 24°, is relatively constant until 40°, and then decreases.

At a lower buffer concentration (20 mM ethanolamine-HCl) with DPN, the activity continues to increase up to 47°, then falls off; the curve is similar to the one shown for TPN in Figure 6.

Effect of DPNH and TPNH on Activity. Figure 7 shows the effect of DPNH on double-reciprocal plots of velocity *vs.* 3,5-diaminohexanoate concentration; uncompetitive inhibition is observed. When the concentration of DPN was varied in the presence of fixed levels of DPNH and 3,5-diaminohexanoate, competitive inhibition was observed; the K_i for DPNH was 19.5 μM. With TPNH, a K_i value of 0.5 μM was found.

Figure 7 shows that at high 3,5-diaminohexanoate concentrations substrate inhibition is observed.[2] By plotting 1/velocity *vs.* 3,5-diaminohexanoate concentration (in the absence of DPNH), K_s, for the second molecule of substrate was determined to be 7.7 mM at pH 7.4 and 28 mM at pH 8.8. Since 3,5-diaminohexanoate solutions were prepared by neutralizing crystalline 3,5-diaminohexanoate-2HCl to pH 6–7, Cl^- is probably partly responsible for the observed inhibition, especially at pH 8.8 where there is no V_{max} effect with increasing Cl^- and high 3,5-diaminohexanoate concentrations are required to see inhibition. It is unlikely that chloride causes all the inhibition because at pH 7.4 inhibition is observed at chloride concentrations that are not very inhibitory.

Inhibition by Nucleotides. ATP was found to be a competitive inhibitor with respect to both 3,5-diaminohexanoate and DPN. When 3,5-diaminohexanoate was the variable substrate, the K_i for ATP was 1.0 mM; with DPN as the variable substrate, the K_i for ATP was 4.5 mM.

Other nucleotides were also effective inhibitors; GTP was as effective as ATP, ADP, and CTP were about half as effective, while AMP and UTP were 32 and 16% as effective as ATP.

Attempts to desensitize the enzyme to ATP inhibition were unsuccessful. The following methods were employed: heat

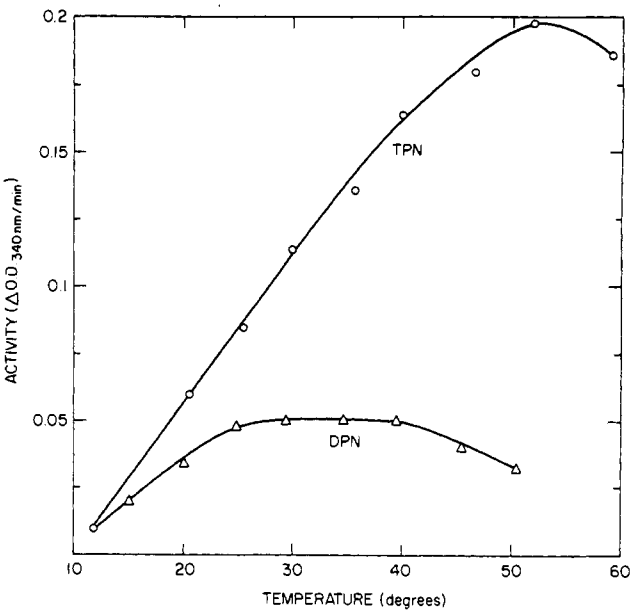

FIGURE 6: Effect of temperature on dehydrogenase activity with TPN or DPN as coenzyme. Activity was determined in the standard assay mixture with 0.62 μg of protein/ml at the indicated temperatures.

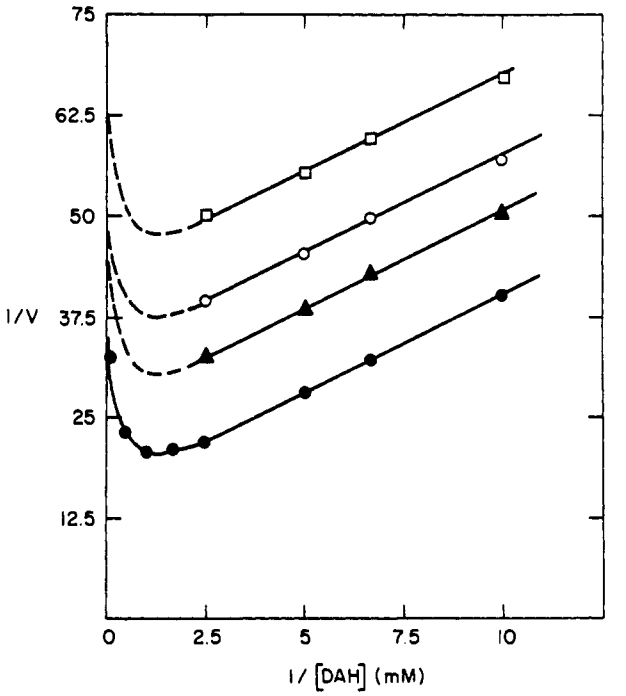

FIGURE 7: Effect of DPNH on double-reciprocal plots of activity (ΔOD/min) *vs.* DAH concentration. Experiments were performed with 0.87 μg of protein 88 mM triethanolamine-HCl (pH 7.4) in the presence of none (●), 42 μM (▲), 70 μM (○), and 127 μM (□) DPNH. The DPN concentration was held constant at 0.4 mM while the DAH concentration was varied as indicated.

[2] Although the D isomer is a good competitive inhibitor, use of DL-*erythro*-3,5-diaminohexanoate as the substrate cannot cause the substrate inhibition because the two isomers are always added in equal amount. The effect of the D isomer is to change the $1/V$ intercept by a factor of $(1 + K_m/K_i)$; the slope does not change.

treatment for 10 min at 50°, incubation for 30 min at both pH 5 and 10.5, treatment with 8 M urea at pH 6.8 for 30 min, reaction with pCl-HgBzO (0.3 μM), assay at either pH 5 or 10.5 and assay in the presence of 0.5 M KCl.

Other Inhibitors. The phosphate and CoA esters of acetate and the final products of the fermentation of L-lysine also were tested as potential inhibitors of dehydrogenase activity. The conditions were the same as those of Table VIII except the indicated compound was used instead of a nucleotide. Acetate (10 mM), butyrate (10 mM), acetyl phosphate (2 mM), or acetyl-CoA (0.62 mM) had no effect. NH_4Cl (10 mM and higher) inhibited the reaction, probably due to the effect of chloride on the activity.

Discussion

Subsequent studies showed that the standard assay conditions used during the purification of the dehydrogenase were not optimal. The assay is significantly improved by substitution of TPN for DPN, which increases the final specific activity from 15.9 to 41.3 units per mg. An even higher value would be obtained if arsenate buffer were used instead of ethanolamine.

Since essentially homogenous preparations of the enzyme are obtained after a 102-fold purification, the dehydrogenase must constitute about 1% of the total soluble protein of *C. sticklandii*. Upon storage, the enzyme slowly polymerizes, especially when it is frozen. Since treatment of the polymerized protein with 6 M urea and dithiothreitol results in a partial restoration of the activity, it is probable that oxidation of sulfhydryl groups is involved in the polymerization. The presence of exposed sulfhydryl groups on the dehydrogenase is also indicated by its sensitivity to inhibition by pCl-HgBzO.

The enzyme is specific for L-*erythro*-3,5-diaminohexanoate. Since the lactam is inactive, the open chain form of L-*erythro*-3,5-diaminohexanoate is the actual substrate. As shown in Table II, structurally related compounds are good competitive inhibitors. Their relative effectiveness provides some knowledge about the substrate binding site. The substrate and the three most effective inhibitors, D-*erythro*-3,5-diaminohexanoate, 2,5-diaminohexanoate, and 2,4-diaminopentanoate have a terminal $CH_3CH(NH_2)CH_2$ group as a common structural feature. The fact that 2,4-diaminopentanoate is a better inhibitor than D- or L-lysine, L-β-lysine, or L-ornithine, indicates that this configuration is more important than the correct carbon chain length.

Deamino-DPN and acetylpyridine-DPN are much poorer substrates than DPN or TPN. The studies of Sarma *et al.* (1968) indicate that in solution deamino-DPN exists in an open rather than stacked ring conformation. The high K_m for deamino-DPN may mean that the dehydrogenase preferentially binds pyridine nucleotides that have a stacked ring conformation. This possibility has been suggested by Eby and Kirtley (1971) for glyceraldehyde-3-phosphate dehydrogenase. Acetylpyridine-DPN which exists in the stacked conformation has a reasonable K_m but its V_{max} is low.

There are several possible explanations for the biphasic kinetics observed with pyridine nucleotides. The enzyme could have a random mechanism, contain two binding sites for the pyridine nucleotides, be a mixture of two enzyme forms or have negative interactions between the two subunits. These possibilities are experimentally distinguishable. The product inhibition pattern shown in Figure 7 is not compatible with a simple rapid equilibrium random mechanism. Although special cases of random mechanisms are possible, the mechanism is most likely ordered with pyridine nucleotide binding first (Cleland, 1963). A complete product inhibition study and binding study are required to conclusively elucidate the mechanism. The presence of more than one binding site for pyridine nucleotides or negative cooperativity can be distinguished by binding studies. These are currently being carried out. The last possibility, a mixture of two forms of the enzyme, can be proven by isolating and characterizing each form. A form of the enzyme, which differs from the one described here in that double-reciprocal plots of activity *vs.* TPN concentration give a straight line, has recently been isolated and will be the subject of a separate investigation. Further studies are required to determine whether a mixture of this recently isolated form and a second form (as yet undetected) cause the biphasic kinetics.

The apparent K_m and V_{max} values appear to be pH dependent. This could be due to loss of a proton from the enzyme or substrates or both. In the case where a proton is lost from a group (or groups) on the enzyme, an unfavorable electrostatic interaction could result. The effect of divalent cations might then be abscribed to neutralization of this charge which would result in a conformation favorable to catalysis. With this scheme divalent cations would have no effect on the enzyme at pH 7.4 because the critical proton is not lost.

The basis of the observed effect of specific ions on the V_{max} and K_m values of the enzyme is not known. The V_{max} effect could be due simply to a general counterion effect in which ions interact with a charged group(s) on the enzyme and either stabilize a conformation optimal for catalysis or favorably shift the pH–velocity curve. The K_m effect is caused by anions which either bind to the pyridine nucleotide site or to a second site which interacts in a competitive manner with the pyridine nucleotide site.

Different anions can have different effects on the dehydrogenase. For example, phosphate and arsenate ions are not as inhibitory as chloride. In their presence V_{max} values are higher (especially for DPN) and K_m values are lower (especially for TPN). Divalent cations have no effect on activity in arsenate at pH 9.1 and there may be little or no pH effect in arsenate because the slight increase in K_m values for DPN between pH 7.4 and 9.1 could be due to differences in ionic strength rather than pH. These differences are difficult to explain unless phosphate and arsenate bind to a different site on the enzyme from chloride. This possibility is supported by the observation that Cl^- is inhibitory in the presence of these buffers as well as in ethanolamine-HCl buffers. A full understanding of these effects will require a much more detailed study with several anions.

Since a relatively sharp temperature optimum is observed at 47° in 20 mM ethanolamine-HCl with DPN as coenzyme, the broad optimum observed in 100 mM ethanolamine-HCl (Figure 6) probably results from an exothermic binding of DPN to the enzyme and the fact that the concentration of DPN used in this experiment (0.4 mM) is well below its K_m (3.3 mM).[3] Consequently, an increase in temperature would result in decreased binding of DPN which could counteract the positive effect of temperature on V_{max} and cause the reaction rate to stay about the same. An alternative explanation is that the positive effect of increasing temperature on V_{max} is counteracted by denaturation of the enzyme and that denaturation occurs in 100 mM ethanolamine, but not 20 mM.

[3] Since the K_m for DPN is considerably lower in 20 mM ethanolamine (1.0 mM); temperature should have less effect on the binding of DPN than it does in 100 mM ethanolamine where the K_m is 3.3-fold larger.

If the data showing the effect of Mn^{2+} concentration on activity of the dehydrogenase are plotted in a Lineweaver–Burk plot, a concave downward curve is obtained. Actually, nonlinear kinetics should be expected because, when Mn^{2+} is not saturating, there will be a mixture of two forms of the enzyme (E and $E \cdot Mn^{2+}$) which are both active and have different K_m and V_{max} values. Since divalent cations have no effect on activity at pH 7.4, the biphasic behavior observed with pyridine nucleotides cannot be explained by these two enzyme forms.

The inhibition of the enzyme by ATP, which is one of the final products of the overall lysine fermentation, is competitive with respect to both the substrate and pyridine nucleotide. Such behavior is commonly observed for allosteric inhibitors and since ATP is not an analog of 3,5-diaminohexanoate, it is likely that it acts allosterically.

The feedback inhibition by ATP and product inhibition by DPNH indicate that the dehydrogenase may play a central role in regulating the rate of lysine fermentation. The severe product inhibition ensures that the rate of reduced pyridine nucleotide formation balances with the availability of electron acceptors. Similarly, the inhibition by ATP ensures that the rate of formation of the high energy product of lysine fermentation is balanced with its utilization for cell materials.

The 3,5-diaminohexanoate dehydrogenase from *C. sticklandii* is remarkably different from the *Clostridium* SB_4 dehydrogenase (Baker *et al.*, 1972) in both physical and kinetic properties. The *C. sticklandii* enzyme is eluted earlier than the SB_4 enzyme from DEAE-cellulose and from hydroxylapatite. The native form is a dimer which has a molecular weight of 80,000 whereas the SB_4 dehydrogenase is a tetramer of molecular weight 140,000. However, the latter readily dissociates to a catalytically active dimer at low ionic strength or pH values above neutrality.

The *C. sticklandii* dehydrogenase differs kinetically from the *Clostridium* SB_4 enzyme in the following properties. (1) It uses both DPN and TPN; the SB_4 enzyme is specific for DPN. (2) It exhibits biphasic kinetics for the pyridine nucleotides. (3) It is activated by divalent cations at high pH. (4) It is very sensitive to certain anions. (5) It is sensitive to feedback inhibition by nucleotides such as ATP.

The two enzymes are similar in a few properties. Both are specific for L-*erythro*-3,5-diaminohexanoate. They have similar product inhibition patterns, pH optima, and both are inhibited at high 3,5-diaminohexanoate concentrations.

Acknowledgments

The authors are greatful to Dr. T. C. Stadtman for encouragement and suggestions on the preparation of the manuscript.

References

Andrews, P. (1965), *Biochem. J. 96*, 595.

Baker, J. J. (1970), Ph.D. Dissertation, University of California, Berkeley.

Baker, J. J., and Barker, H. A. (1970), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29*, 343.

Baker, J. J., Jeng, I. M., and Barker, H. A. (1972), *J. Biol. Chem. 247*, 7724.

Baker, J. J., van der Drift, C., and Stadtman, T. C. (1973), *Biochemistry 12*, 1054.

Cleland, W. W. (1963), *Biochim. Biophys. Acta 67*, 104.

Eby, D., and Kirtley, M. E. (1971), *Biochemistry 10*, 2677.

Hedrick, J. L., and Smith, A. J. (1968), *Arch. Biochem. Biophys. 126*, 155.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Nielsen, N. C., Zahler, W. L., and Fleischer, S. (1973), *J. Biol. Chem. 248*, 2556.

Sarma, R. H., Ross, V., and Kaplan, N. O. (1968), *Biochemistry 7*, 3052.

Stadtman, T. C. (1966), *Arch. Biochem. Biophys. 113*, 9.

Tiselius, A., Hjerten, S., and Larsen, O. (1956), *Arch. Biochem. Biophys. 65*, 132.