Oxidized Triphosphopyridine Nucleotide Specific Isocitrate Dehydrogenase from *Azotobacter vinelandii*. Isolation and Characterization*

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ABSTRACT: An oxidized triphosphopyridine nucleotide specific isocitrate dehydrogenase has been isolated from *Azotobacter vinelandii* (ATCC 9104) cells. The enzyme is obtained in yields of 15–20% from unfractionated cell extracts. The purified enzyme appears to be homogeneous by the criteria of disc gel electrophoresis, ultracentrifugation, and gel filtration. It catalyzes the reduction of 120–135 μmoles of oxidized triphosphopyridine nucleotide/min per mg of protein under the assay conditions used. The purified enzyme is stable for several months in the frozen state. The enzyme has a molecular weight of 80,000, an $s^0_{20,w}$ of 4.6×10^{-13} sec, a $D_{20,w}$ of 5.4×10^{-7} cm^2 sec^{-1}, and an axial ratio of 5.0. By optical rotatory dispersion and circular dichroism measurements the native enzyme appears to have an α-helix content of about 30%. The enzyme does not dissociate into subunits in 6 M guanidine hydrochloride in the presence of reducing agents. The amino acid composition of the enzyme has been determined. It appears to have only three half-cysteine residues per mole of enzyme. The three thiol residues exhibit different degrees of reactivity. One of the thiol residues is readily titrated by 5,5′-dithiobis(2-nitrobenzoic acid), iodoacetic acid, and *p*-hydroxymercuribenzoate. The titration of this reactive thiol group results in a complete loss of catalytic activity. The reactivity of a second thiol group is enhanced by titration of the first with *p*-hydroxymercuribenzoate. The third thiol residue appears to be buried within the protein molecule and is slowly titrated by *p*-hydroxymercuribenzoate. This thiol group becomes more reactive if the native enzyme is denatured with guanidine hydrochloride. The enzyme shows a high degree of specificity for oxidized triphosphopyridine nucleotide as electron acceptor; oxidized diphosphopyridine nucleotide has less than 1% of the activity of oxidized triphosphopyridine nucleotide for accepting hydrogens from isocitrate. Oxidized deaminotriphosphopyridine nucleotide is incapable of serving as a substrate in the enzyme reaction. The purified enzyme is not markedly affected by a variety of adenine nucleoside derivatives which affect the oxidized diphosphopyridine nucleotide specific isocitrate dehydrogenases. The Michaelis constants for oxidized triphosphopyridine nucleotide and *threo*-D_s-isocitrate are 2.3×10^{-5} and 2.0×10^{-5} M, respectively.

Isocitrate dehydrogenases which utilize either TPN$^+$ or DPN$^+$, respectively, as coenzymes are found in a wide variety of tissues and organisms (Plaut, 1963). The central role which these enzymes play in energy production and generation of reduced pyridine nucleotide coenzymes has prompted intensive studies on their properties and their mode of regulation (Plaut, 1963; Atkinson *et al.*, 1965; Sanwal and Stachow, 1964; Plaut and Aogaichi, 1968). Furthermore, the dual catalytic activities, *viz.*, dehydrogenation and decarboxylation, which they exhibit provide interesting models for detailed analysis of the mechanism of multifunctional enzymes.

It is well established that the DPN$^+$- and TPN$^+$-specific enzymes differ in their catalytic properties, stability, and mode of regulation (Plaut, 1963; Stadtman, 1966). Evidence is now accumulating which indicates that even different TPN$^+$-specific enzymes exist (Henderson, 1968). Recently, Marr and Weber (1968) reported that the TPN$^+$-specific enzyme from *Salmonella typhimurium* was inhibited by purine nucleoside di- and triphosphates. These effects are not observed with other TPN$^+$-specific enzymes. The marked differences which exist between different isocitrate dehydrogenases may reflect the particular role that each enzyme plays in the over-all economy of the biological system from which it is derived. The molecular bases for the differences, however, are completely unknown.

In spite of the wide occurrence of the isocitrate dehydrogenases, the TPN$^+$-specific enzyme from pig heart is the only one which has been thoroughly characterized with respect to its physical and chemical properties (Plaut, 1963). With the over-all view of understanding the detailed chemical mechanism of action of isocitrate dehydrogenase and the regulation of its activity and synthesis we undertook the task of isolating and characterizing the TPN$^+$-specific enzyme from *Azotobacter vinelandii* (ATCC 9104) cells. The initial results of these experiments are reported in this communication.

Experimental Procedure

Materials. All nucleotides and coenzymes, *threo*-D_sL_s-isocitrate trisodium salt, *threo*-D_s-isocitrate trisodium salt, DTNB,[1] DTT, PMB, and α-ketoglutarate were purchased from Sigma. DEAE-cellulose and CM-cellulose were ob-

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[1] Abbreviations used are: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; PMB, *p*-hydroxymercuribenzoate; IAA, iodoacetic acid.

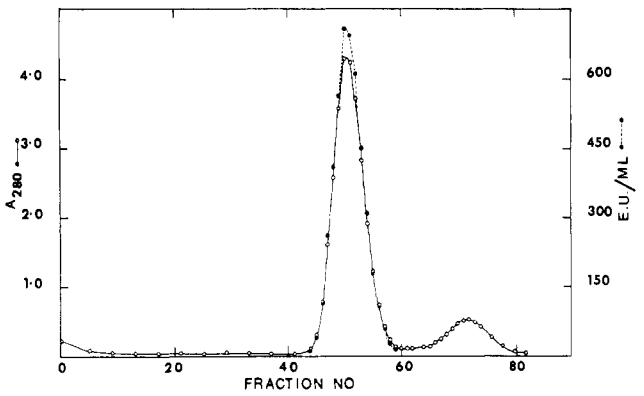


FIGURE 1: Sephadex G-100 fractionation of isocitrate dehydrogenase. Simultaneous patterns of absorbancy at 280 nm and enzyme activity per ml against fraction number are presented. The eluting buffer was 0.1 M potassium phosphate buffer (pH 7.0).

tained from Carl Schleicher und Schuell. Sephadex G-25 and G-100 were obtained from Pharmacia. Enzyme grade ammonium sulfate was supplied by Mann Research Laboratories. The barium salt of oxalosuccinic acid was obtained from Nutritional Biochemicals. Eastman Organic Chemicals supplied acrylamide, *N,N'*-methylenebisacrylamide, *N,N,N',N'*-tetramethylethylenediamine, and glycine. [1-^{14}C]IAA and [1-^{14}C]iodoacetamide were obtained from Tracerlab, Waltham, Mass. All other reagents were analytical grade obtained from various commercial sources.

Methods. DEAE-cellulose and CM-cellulose were prepared as previously described (Mize and Langdon, 1962). In the early stages of enzyme purification protein was determined by the method of Gornall *et al.* (1949) with bovine serum albumin as standard. In the purified state the protein concentrations was determined by its absorbancy at 280 nm. It was determined that $A_{280}^{1\%}$ was 8.9 by redissolving a dried weighed sample of the homogeneous enzyme in water. All steps in the purification were conducted at 0–4° and glass-distilled water was used throughout for preparation of buffers and reagents.

Assay of the Enzyme. Unless otherwise indicated, the enzyme activity was measured at 30° in 0.05 M potassium phosphate buffer (pH 7.0) with 10^{-4}, 4×10^{-3}, and 5×10^{-3} M TPN^+, DL-isocitrate, and magnesium sulfate, respectively, in a total volume of 2.0 ml. The reaction was initiated with 5 or 10 μl of enzyme solution which had been appropriately diluted. The initial velocity was measured by the rate of absorbancy at 340 nm over the first 2 min of the reaction. One enzyme unit is defined as the amount of enzyme which catalyzes the reduction of 1 μmole of TPN^+ in 1 min under the above assay conditions. The specific activity is expressed in enzyme units per milligram of protein.

Amino Acid Determination. Enzyme samples were hydrolyzed in 6 N HCl in sealed tubes under nitrogen at 110° for 20 hr or 60 hr. The amino acids obtained by hydrolysis were analyzed with a Spinco Model 116 amino acid analyzer according to the method of Spackman *et al.* (1958). The tryptophan content of the enzyme was determined by the spectrophotometric method of Goodwin and Morton (1946). The cysteine content of the enzyme was determined on the hydrolysate of the enzyme which had been treated with performic acid as described by Hirs (1967). The cysteine content was further determined by titration with PMB according to the method of Boyer (1954) and by titration with DTNB as described by Ellman (1959).

Ultracentrifugation Analysis. Measurements of sedimentation velocity, diffusion, and molecular weight by equilibrium methods were carried out with the Beckman-Spinco Model E analytical ultracentrifuge. For sedimentation and diffusion measurements, the schlieren optical system was employed, and the temperature was controlled at either 4 or 20°. An apparent specific volume of 0.74 $cm^3\ g^{-1}$, calculated from the amino acid composition, was used for computations. Equilibrium centrifugations using interference optics were performed both by the low-speed and high-speed methods (Van Holde and Baldwin, 1958; Yphantis, 1964). All equilibrium centrifugations on the native enzyme were carried out on samples that had been exhaustively dialyzed against solvent, 0.05 M potassium phosphate buffer at pH 7.0. The dialysate was present in the reference channel. Halocarbon oil, series 11–14 (Halocarbon Products Corp., Hackensack, N. J.), was present at the base of each channel. The optics were aligned with the camera lens focused on the midplane of the cell (Gropper, 1964). For high-speed experiments on the native enzyme, weight average molecular weights were determined from the least-squares-fitted line through the points of a graph of the logarithm of the fringe displacement *vs.* radial distance squared. All such plots were linear. Weight-average molecular weights determined at regularly spaced intervals across the concentration gradient by the five-point-unweighted least-squares procedure of Yphantis showed no trend in the values as a function of radial position.

The high-speed equilibrium centrifugation of the enzyme in 6 M guanidine hydrochloride containing 0.01 M DTT was carried out for 70 hr at 20°. The sample had been exhaustively dialyzed against the solvent, and the dialysate was used in the reference channel. A value for the apparent specific volume of 0.73 $cm^3\ g^{-1}$ was employed for the calculation of molecular weights in this case, based on the considerations of apparent specific volumes of proteins in guanidine hydrochloride reported by Noelken and Timasheff (1967) and Hade and Tanford (1967).

Optical Rotatory Dispersion and Circular Dichroism Spectra. Optical rotatory dispersion and circular dichroism spectra were determined with a Cary 6002 recording spectropolarimeter equipped for the measurement of circular dichroism. The spectra were determined at a nominal temperature of 25°. Spectra in the region above 200 nm were obtained in 0.05 M phosphate buffer at pH 7, while those below 200 nm were obtained in water with no added salt. Solvent blanks were run to establish the proper base lines. The optical rotatory dispersion spectra from 600 to 380 nm were analyzed in terms of the Moffitt–Yang and Schechter–Blout parameters. A value for λ_0 of 212 nm was used for the Moffitt–Yang analysis.

Enzyme Purification. PREPARATION OF *A. vinelandii* EXTRACTS. *A. vinelandii* cells were grown aerobically at 30° for 48 hr in 9-l. bottles on Burks nitrogen-free medium (Wilson and Knight, 1952) to a turbidity level of 200–250 Klett units (no. 66 filter). The cells were harvested with a refrigerated Sharples centrifuge. The harvested cells (400 g wet weight) were suspended in three volumes of 1 M glycerol in 0.02 M potassium phosphate buffer (pH 7.0). Portions (400 ml) of the cell suspension were sonically treated for 10 min in a Heat

TABLE I: Purification of Isocitrate Dehydrogenase.

Fraction	Total Vol (ml)	Total Protein (mg)	Total Enzyme (units)	Sp Act. (units/mg)	Recovery (%)
1. Sephadex G-25	1,800	54,000	81,000	1.5	100
2. DEAE-cellulose	3,600	33,840	75,600	2.2	93
3. Gel supernatant	4,000	28,000	72,000	2.6	89
4. Ammonium sulfate	50	1,000	35,700	35.7	44
5. CM-cellulose	7.4	182.8	17,745	97.0	22
6. Sephadex G-100	4.5	131.4	17,460	133.0	22

Systems sonicator at 6–8 A. The sonically treated suspension was centrifuged at 30,000 rpm in a Spinco no. 30 rotor for 30 min. The supernatant solution was used as the source of enzyme.

DEAE-CELLULOSE FRACTIONATION. Crude extract (1000 ml) was filtered through a Sephadex G-25 column (60 × 10 cm) to remove low molecular weight compounds. The enzyme was eluted in 0.05 M potassium phosphate buffer containing 1.5×10^{-5} M DPN^+ which had been used to equilibrate the Sephadex column. The eluate containing the enzyme was applied to a DEAE-cellulose column (37 × 10 cm) which had previously been equilibrated with 0.02 M potassium phosphate buffer containing 1.5×10^{-5} M DPN^+. A colored band of protein which contained the enzyme was eluted from the column with 0.05 M potassium phosphate buffer (pH 7.0) containing 1.5×10^{-5} M DPN^+. DPN^+ was added to the buffers because the extract was also used for the isolation of NAD^+ kinase which is stabilized by this coenzyme. For isocitrate dehydrogenase the DPN^+ may be omitted.

CALCIUM PHOSPHATE GEL FRACTIONATION. Calcium phosphate gel at a gel to protein ratio of 1:1 was added to the eluate from the DEAE-cellulose fractionation. After 10 min the suspension was centrifuged at 9000 rpm in a Sorvall GSA rotor for 10 min. The supernatant solution which contained the enzyme was next fractionated with ammonium sulfate.

AMMONIUM SULFATE FRACTIONATION. To each liter of enzyme solution 351 g of ammonium sulfate and 0.7 ml of 2-mercaptoethanol were added. After standing for 30 min the suspension was centrifuged for 15 min at 9000 rpm in a Sorvall GSA rotor. The precipitate was discarded and an additional 211 g of ammonium sulfate/l. of original enzyme solution was added to the supernatant solution. After standing for 60 min the precipitate was collected by centrifugation as described previously. The supernatant solution was discarded and the precipitate was dissolved in approximately 50 ml of 0.05 M potassium phosphate buffer (pH 7.0). This solution contained the enzyme.

CM-CELLULOSE FRACTIONATION. The enzyme solution obtained from the ammonium sulfate fractionation was filtered through a Sephadex G-25 column (4.5 × 47 cm). The enzyme was eluted in 2.5×10^{-3} M potassium phosphate buffer (pH 6.2) containing 10^{-3} M 2-mercaptoethanol which had been used to equilibrate the Sephadex column. After this step the solution usually became somewhat turbid. The eluate was clarified by centrifugation at 10,000 rpm in a Sorvall SS-34 rotor for 10 min. The supernatant solution was applied to a CM-cellulose column (2 × 45 cm) which had been previously equilibrated with 2.5×10^{-3} M potassium phosphate buffer (pH 6.2) containing 10^{-3} M 2-mercaptoethanol. After application of the enzyme solution, the column was percolated with the starting buffer until the absorbancy of the effluent solution was about 0.050 at 280 nm. At this point the column was eluted with 0.01 M potassium phosphate buffer (pH 6.2), containing 10^{-3} M 2-mercaptoethanol and 3×10^{-3} M DL-isocitrate. Fractions of approximately 8 ml each were collected and assayed for enzyme activity and protein content. The fractions which contained the enzyme with highest specific activity were pooled and concentrated by ultrafiltration in an atmosphere of nitrogen with an Amicon diaflo apparatus (Amicon Corp. Mass.). A UM-10 filter was used.

SEPHADEX G-100 FRACTIONATION. The concentrated enzyme solution was finally fractionated on a column of Sephadex G-100 (2.5 × 91 cm) which had been equilibrated with 0.1 M potassium phosphate buffer (pH 7.0). Fractions of approximately 4 ml were collected and assayed for protein and enzyme. The elution patterns of protein and enzyme activity are shown in Figure 1. The fractions which contained the enzyme were pooled and concentrated by ultrafiltration.

The results of a typical purification scheme are summarized in Table I.

Results

Purified Enzyme Preparation. The purified enzyme preparations consistently exhibited specific activities between 120 and 135 enzyme units per mg of protein. The yields obtained varied between 15 and 30% of the original activity present in the unfractionated extracts. The enzyme preparations obtained were homogeneous as judged by disc gel electrophoresis on acrylamide columns, ultracentrifugation, and gel filtration on Sephadex G-100. Figures 2 and 3 show typical patterns obtained for the enzyme on the acrylamide gel and in the ultracentrifuge, respectively. The specific activity of the enzyme band obtained by gel filtration on Sephadex G-100 was constant throughout the elution profile as shown by the superposition of the protein and enzyme elution profiles in Figure 1.

Amino Acid Composition. The amino acid composition of the enzyme is shown in Table II. One of the more interesting features of the amino acid composition is the low content of cysteine residues. The enzyme contains 3 cysteine residues/80,000 g of protein. The enzyme does not contain any disulfide bonds as indicated by titration of all three sulfhydryl groups with PMB and with DTNB in 4 M guanidine hydrochloride. Titration of the enzyme with excess PMB yielded an

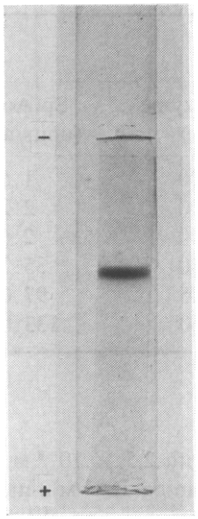


FIGURE 2: Acrylamide gel electrophoresis of isocitrate dehydrogenase. The acrylamide gel electrophoresis was carried out by the method of Ornstein (1964) and Davis (1964), with a Buchler Polyprep electrophoresis apparatus. The samples used contained 29 μg of isocitrate dehydrogenase, specific activity 130 enzyme units/mg of protein. The electrophoresis was carried out at a constant current of 2.5 mA/tube for 90 min at pH 9 in Tris-HCl buffer. The acrylamide gel used was the standard gel with 7.5% cross-linkage. The gel was stained for protein with coomassie blue according to the method described by Chrambach *et al.* (1967).

average of 3.2 moles of sulfhydryl groups/80,000 g of protein. Titration with DTNB in 4 M guanidine hydrochloride yielded 2.8 moles of sulfhydryl groups/80,000 g of enzyme.

Physical Parameters of the Enzyme. The results of the diffusion, sedimentation velocity, and sedimentation equilibrium studies are summarized in Table III. The observed constancy of weight-average molecular weight values at all points in the cell during the high-speed equilibrium centrifugation experiments and the agreement between number-average and weight average molecular weights calculated from the Yphantis-type ultracentrifuge runs further demonstrate the homogeneity of the preparation. Figure 4 illustrates representative high-speed equilibrium ultracentrifuge data on the native enzyme and on the enzyme in a solvent which should fully denature the protein and dissociate it into its constituent subunits if such exist. It is clear from this figure that the protein has the same molecular weight under dissociating conditions as it does in the native state. At higher protein concentrations, toward the base of the ultracentrifuge cell, the apparent molecular weight is reduced relative to its value in the very dilute concentration range. The value of the second virial coefficient calculated for isocitrate dehydrogenase in guanidine hydrochloride from the data in Figure 4 is $60 \pm 5 \times 10^{-5}$ cm^3 mole g^{-2} which is in the range of values reported for other proteins in this solvent (Castellino and Barker, 1968). There is nothing unusual therefore about the negative curvature of the lower set of points in Figure 4.

The optical rotatory dispersion and circular dichroism curves of the native enzyme are portrayed in Figure 5, and the significant parameters describing these curves and the extension of the optical rotatory dispersion spectrum to the visible range are registered in Table IV. On the basis of the

TABLE II: Amino Acid Composition of Isocitrate Dehydrogenase.

Amino Acid	Residues/80,000 g of Protein[a]
Aspartic acid	70
Threonine[b]	39
Serine[b]	42
Glutamic acid	61
Proline	33
Glycine	45
Alanine	76
Half-cystine[c]	3
Valine	42
Methionine	8
Isoleucine	39
Leucine	60
Tyrosine	19
Phenylalanine	17
Tryptophan[d]	7
Lysine	52
Histidine	13
Arginine	24

[a] The values reported are the mean values obtained from three separate and independent experiments except as indicated. In each experiment the amino acid composition was determined in duplicate. The results are expressed to the nearest integer. Two samples were digested for 20 hr and one sample for 60 hr in 6 N HCl at 110°. [b] These values were corrected to zero time of hydrolysis. [c] The half-cystine content was determined by three separate methods: (i) determination as cysteic acid after performic acid oxidation, (ii) titration with DTNB, and (iii) titration with PMB. The mean value is given. [d] Determined spectrophotometrically.

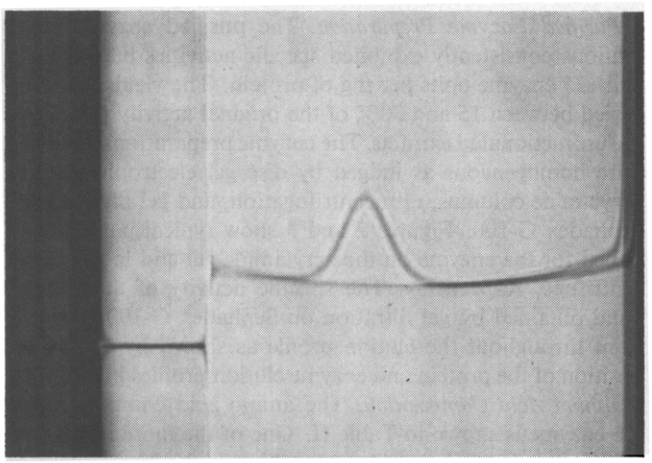


FIGURE 3: Sedimentation pattern of isocitrate dehydrogenase. The centrifugation was carried out at 4° in 0.10 M potassium phosphate buffer (pH 7.0). The picture was taken after centrifugation at 59,780 rpm for 89 min. The specific activity of the enzyme was 133 enzyme units/mg of protein, and the protein concentration was 8.4 mg/ml.

TABLE III: Physical Parameters of *A. vinelandii* Isocitrate Dehydrogenase.

Parameter	Value
$s^0_{20,w}$ (sec)	4.57×10^{-13}
$D_{20,w}$ (cm^2 sec^{-1})	5.4×10^{-7}
Molecular weight (s/D)	78,400
Molecular weight (Yphantis)	81,400
Molecular weight (Yphantis, 6 M guanidine hydrochloride–0.01 M DTT)	81,000
Molecular weight (Van Holde–Baldwin)	77,300
Axial ratio for a prolate ellipsoid with a solvation factor of 0.2 g/g	5.0 (from D)

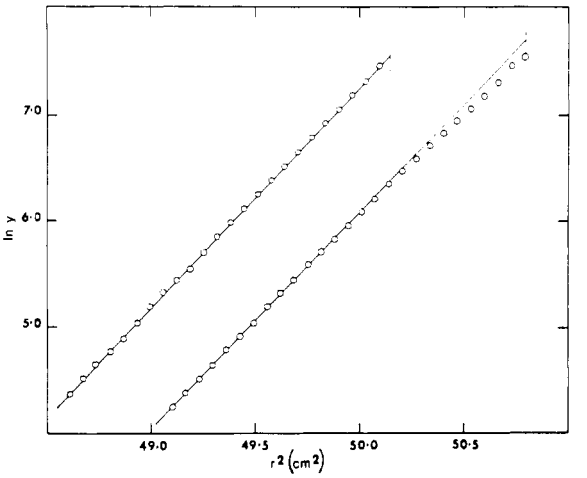


FIGURE 4: Display of high-speed equilibrium centrifuge data on native and denatured isocitrate dehydrogenase. The upper line represents the natural logarithm of the fringe displacements, measured in microns, as a function of the square of the radial distance for the native protein. The lower line corresponds to the same quantities for the protein in 6 M guanidine hydrochloride–0.01 M DTT. The measured values of ln y for the guanidine hydrochloride data have been multiplied by the factor $[(1 - \bar{v}\rho)/(1 - \bar{v}\rho)'](rpm/rpm')^2(T'/T)$, where the primed terms employ values corresponding to the run in guanidine hydrochloride and the unprimed terms employ values corresponding to the standard solvent run. The vertical bar at the right-hand terminus of each line represents the position of the base o the liquid column.

optical rotatory dispersion-circular dichroism data about one-third of the peptide backbone residues can be provisionally assigned to the α-helical conformation. Probably the most definitive indication of the existence of some helical component is the double minimum in the circular dichroism spectrum, which shows minima at the characteristic 222- and 209-nm positions.

Role of Sulfhydryl Groups in the Catalytic Activity of the Enzyme. The enzyme is strongly inhibited by sulfhydryl reagents such as PMB, IAA, DTNB, and *N*-ethylmaleimide. Reaction of the enzyme with IAA and DTNB indicates that titration of one of the three sulfhydryl groups of the native enzyme results in almost complete loss of catalytic activity. This essential sulfhydryl group is more reactive than the other two sulfhydryl groups. Inactive enzyme, prepared by titration of one sulfhydryl group per mole of enzyme with DTNB, can be almost completely reactivated by removal of the thionitrobenzoic acid group from the enzyme with DTT. The results of experiments with DTNB are summarized in Table V. Treatment of the catalytically inactive DTNB-modified enzyme with DTT releases 1 mole of chromophore/mole of enzyme with 95% recovery of enzyme activity. The regenerated enzyme was indistinguishable from the native enzyme with respect to the Michaelis constants for TPN$^+$

TABLE IV: Optical Rotatory Dispersion and Circular Dichroism Parameters of *A. vinelandii* Isocitrate Dehydrogenase.

Parameter	Value (deg cm^2 dmole^{-1})[a]	% Helix[b,c]
a_0	−205	
b_0	−197	31
A_{225}	−696	32
A_{193}	699	40
$A_{193} - A_{225}$		37
$[\phi']_{233}$	−5,400	26
$[\phi']_{198}$	22,000	34
$[\theta']_{222}$	−10,100	34
$[\theta']_{209}$	−8,400	
$[\theta']_{193}$	+17,400	32

[a] All values have been corrected for refractive index using the Lorentz factor. [b] Helix contents based on optical rotatory dispersion measurements are evaluated according to relations compiled by Yang (1967). [c] Helix contents based on ellipticity measurements are evaluated on the basis of reference values compiled by Beychok (1968).

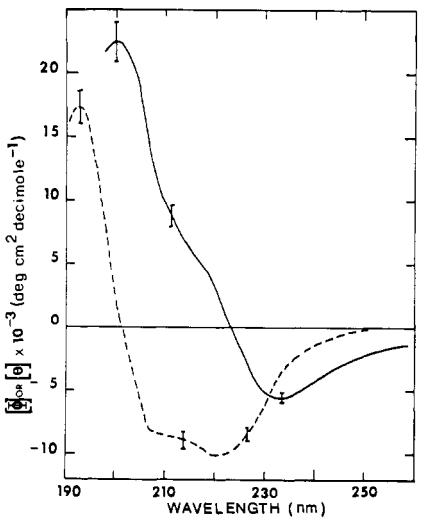


FIGURE 5: Optical rotatory dispersion and circular dichroism spectra of native isocitrate dehydrogenase. Molar rotations, solid line, and molar ellipticities, dashed line, are both corrected by the Lorentz factor using the refractive index of water as an approximation to that of the solvent. The vertical bars approximately indicate amount of uncertainty in the parameters in various regions of the spectra.

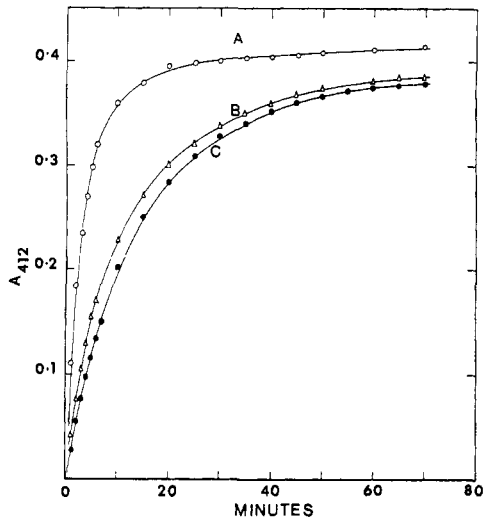
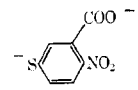


FIGURE 6: Titration of isocitrate dehydrogenase with DTNB in the presence and absence of substrates. The basic reaction mixture containing 3.28 mg of enzyme, specific activity 130 enzyme units/mg of protein, in 2 ml of 0.05 M potassium phosphate buffer (pH 8.0) was placed in a standard cuvet maintained at 30° in a thermostated cuvet holder. To this solution 0.02 ml of DTNB (4 mg/ml of 0.05 M potassium phosphate buffer (pH 7.0)) was added and the absorbancy at 412 nm was recorded with a Zeiss PMQ II spectrophotometer at fixed times. The absorbancies were determined against a blank which contained no enzyme. In curve A the cuvet contained enzyme only, in curve B the cuvet contained enzyme and DL-isocitrate at a concentration of 2×10^{-3} M, in curve C the cuvet contained enzyme and TPN^+ at a concentration of 5×10^{-4} M.

and *threo*-D_s-isocitrate which were 2.3×10^{-5} and 2.0×10^{-5} M, respectively, for both native and regenerated enzyme. The sedimentation constants were identical for both enzyme preparations. The rate of titration of the reactive sulfhydryl group by DTNB was decreased by the addition of either TPN^+ or DL-isocitrate as shown in Figure 6. The initial rate of titration of the enzyme is decreased by more than 50% by the addition of either substrate.

In Figure 7A the loss of catalytic activity is correlated with the binding of [1-^{14}C]IAA. It may be seen that the binding of 1 mole of reagent/mole of enzyme results in approximately 98% loss of catalytic activity. The catalytic activity could not be regenerated by DTT. The titration was carried out at pH 8 which would enhance the reaction of this reagent with thiol residues in the protein molecule. The nonlinear relationship between the loss of catalytic activity and binding of inhibitor at less than a 1:1 ratio of inhibitor to enzyme is not clear at the present time. The native enzyme is stable under the conditions of the experiment in the absence of IAA which rules out a nonspecific loss of catalytic activity. Titration of the enzyme with PMB yielded results which were somewhat different from those obtained with either DTNB or IAA. As shown in Figure 7B the enzyme was not completely inactivated until 2 equiv of mercaptide/mole of enzyme was formed. These results indicate that the three thiol residues in the protein molecule exhibit different reactivities toward different sulfhydryl reagents. One thiol group which is essential for activity is readily titrated with DTNB and IAA. The other two groups are not as readily titrated with these reagents. Two of the three thiol groups are readily titrated with PMB; one of these two groups is probably

TABLE V: Inactivation of Isocitrate Dehydrogenase by DTNB and Reactivation with DTT.[a]

Treatment	Enzyme Units/mg of Protein	Moles of  Bound or Released/Mole of Enzyme
None	126	
+DTNB	5.2	0.91
+DTNB + DTT	111	0.89

[a] Isocitrate dehydrogenase (2.5 ml), containing 19 mμmoles of enzyme/ml of 0.05 M potassium phosphate buffer (pH 7.0), was mixed with 0.02 ml of DTNB (4 mg/ml of 0.05 M potassium phosphate buffer (pH 7.0)) and allowed to stand at 23° for 90 min. At the end of this time the absorbancy at 412 nm had reached a constant value. The net change in absorbancy was used to calculate the number of sulfhydryl residues titrated per mole of enzyme. A value of 13.6×10^3 for the molar absorbance of the chromophore released was used (Ellman, 1959). An aliquot of this mixture was used to determine enzyme activity by the standard assay procedure described in the text. The remainder of the titrated enzyme was freed of excess DTNB and released chromophore by gel filtration on Sephadex G-25. The enzyme solution obtained from the Sephadex G-25 filtration was concentrated by ultrafiltration to a concentration of 9.8 mμmoles of enzyme/ml of 0.05 M potassium phosphate buffer (pH 7.0). To 2.7 ml of this solution 0.3 ml of 0.1 M DTT dissolved in 0.05 M potassium phosphate buffer (pH 7.0) was added. The mixture was allowed to stand at 23° and the absorbancy was determined at fixed time intervals. After 32 min the absorbancy at 412 nm had become constant and the net change in absorbancy (after correction for blank which contained buffer and DTT only) was used to calculate the release of chromophore from the enzyme. The enzyme activity in this solution was determined in the usual manner. The results are shown in the table as indicated by +DTNB + DTT.

that essential for catalytic activity since there is complete loss of activity upon titration with PMB as described here. The third sulfhydryl group of the enzyme is titrated slowly by PMB. The rate of titration of the thiol groups by PMB is shown in Figure 8. The results clearly show that in the presence of excess PMB two thiol groups of the enzyme are titrated in less than 1 min while the third residue is not completely titrated until more than 90 min had elapsed.

Catalytic Properties of the Purified Enzyme. The apparent Michaelis constants for TPN^+ and *threo*-D_s-isocitrate were 2.3×10^{-5} and 2.0×10^{-5} M, respectively, as determined by conventional techniques at 30° (Lineweaver and Burk, 1934). The enzyme appears to have an almost absolute specificity for TPN^+. DPN^+ at concentrations of 10^{-4} and 1.25×10^{-3} M exhibited only 0.06 and 0.48%, respectively, of the activity obtained with TPN^+ at a concentration of 10^{-4} M under other-

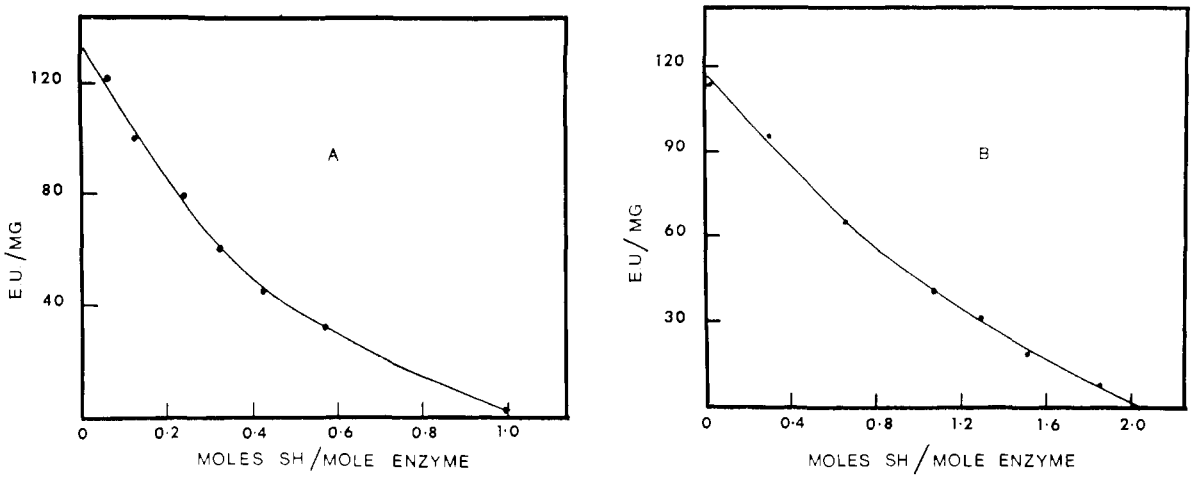


FIGURE 7: Titration of isocitrate dehydrogenase with [1-^{14}C]iodoacetic acid and *p*-hydroxymercuribenzoate. In part A the titration of the enzyme with [1-^{14}C]iodoacetic acid is shown. To 6 ml of enzyme solution in 0.05 M potassium phosphate buffer (pH 8.0) containing 1.1 mg of protein/ml (specific activity 130 enzyme units/mg of protein), 1.6 μmoles of [1-^{14}C]iodoacetic acid with a total radioactivity of 10 μCi was added in a total volume of 0.1 ml of buffer as above. The mixture was incubated at 23°. At predetermined times 0.5 ml of the mixture was removed and unbound radioactivity removed from the enzyme by filtration through a Sephadex G-25 column (1 × 15 cm); 1-ml fractions were collected from the column and the protein concentration was determined in each fraction by its absorbancy at 280 nm. The residual enzyme activity in the peak fraction was determined. Aliquots (0.2 ml) from this fraction were removed for radioactivity measurements. The radioactivity was determined after solution of the protein in 0.2 ml of NCS reagent and addition of 10 ml of Bray's scintillation fluid (Bray, 1960). Radioactivity measurements were carried out with a Beckman LS-100 liquid-scintillation counter. The enzyme specific activity was calculated from the residual enzyme activity and protein concentration. The number of moles of enzyme thiol groups titrated was calculated from the radioactivity bound to the enzyme and the specific activity of the [1-^{14}C]iodoacetic acid. The number of moles of thiol groups titrated per mole of enzyme was calculated from the moles of thiol titrated as determined by radioactivity measurements and the concentration of protein as determined by absorbancy measurements. In part B the titration of the enzyme with PMB is shown. The titration was carried out according to the method of Boyer (1954). To 1.07 ml of enzyme solution in 0.05 M potassium phosphate buffer (pH 7.0) containing 1.16 mg of protein/ml (specific activity 120 enzyme units/mg of protein), 0.010- or 0.005-ml aliquots of 10^{-3} M PMB in buffer as above were added. After each addition of PMB the absorbancy at 250 nm was recorded when it reached a constant value. At this point a 0.005-ml aliquot of the mixture was removed for enzyme assay. Another aliquot of PMB solution was added and the procedure was repeated. The A_{250} readings were measured against a blank which contained the same volume of buffer and which was treated in an identical manner. The temperature of the reaction was maintained at 30°. After the appropriate corrections were made for dilution, the number of thiol groups titrated was calculated from the net change in A_{250}. The specific activity of the enzyme was calculated from the residual activity and the protein concentration.

wise identical assay conditions. The deamino analog of TPN^+ was completely incapable of serving as a substrate at all concentrations tested. The enzyme requires either Mg^{2+} or Mn^{2+} for activity. The replacement of magnesium by 2.5×10^{-3} M EDTA in the standard assay system lowers the activity of the enzyme to less than 0.02% of that in the unmodified assay system. This low activity was detectable only with a large excess of enzyme. Maximal catalytic activity was obtained with Mg^{2+} at concentrations ranging from 6×10^{-4} to 2×10^{-2} M. At higher concentrations a slight inhibition was observed. The optimal concentration of Mn^{2+} was obtained at 10^{-2} M. Higher concentrations had no further effect. In the experiments with Mn^{2+} the assay was carried out in 0.05 M potassium phosphate buffer at pH 7.0. The enzyme catalyzed the oxidation of TPNH in the presence of α-ketoglutarate and bicarbonate under the conditions described by Colman (1968). The rate of oxidative carboxylation was 66% of that observed for the forward reaction with TPN^+ and isocitrate as substrates.

Effect of Nucleotides and Other Compounds on the Catalytic Activity. A variety of nucleotides, DPN^+, and oxaloacetate were tested for their effects on the catalytic activity of the enzyme. The results are summarized in Table VI. The various compounds tested had marginal inhibitory effects on the enzyme activity. In an attempt to exaggerate the slightly inhibitory effects of these compounds the concentration of isocitrate was decreased by a factor of 10. The results were not markedly different as may be seen in Table VI.

Discussion

The experiments which have been described in this communication have yielded some fundamental information on the properties and behavior of isocitrate dehydrogenase isolated from *A. vinelandii* cells. The enzyme occurs in very high concentration in this microorganism and represents approximately 1–2% of the total soluble protein of the cell. This organism therefore is an excellent source for the isolation of this enzyme in substantial quantities in an homogeneous state for detailed analysis of its chemistry, mechanism of action, and mode of regulation. The specific activity of this enzyme is severalfold greater than that hiterto reported for the enzyme isolated from other sources (Plaut, 1963). The highly purified enzyme may be stored for months in the frozen state without loss of activity, which is of great advantage for its characterization.

The enzyme has a molecular weight of approximately 80,000 as determined by a variety of techniques. In this respect it is a large enzyme. It is now recognized that many enzymes which utilize the pyridine nucleotide coenzymes as cofactors can be dissociated into subunits of molecular weight of approxi-

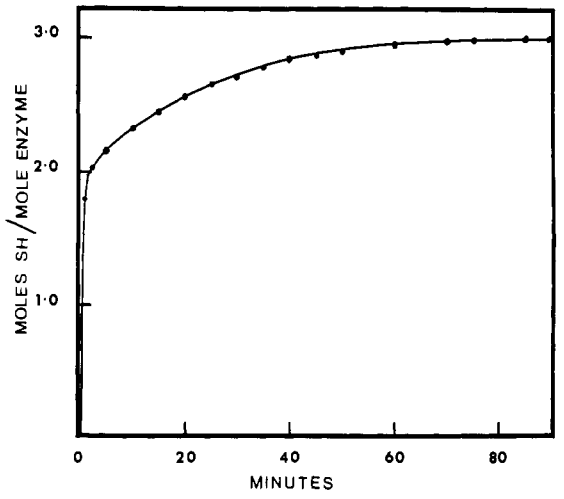


FIGURE 8: Rate of titration of isocitrate dehydrogenase with *p*-hydroxymercuribenzoate. In a standard cuvet 1.5 ml of enzyme (specific activity 130 enzyme units/mg of protein) which contained 4.72 mg of protein/ml of 0.05 M potassium phosphate buffer (pH 7.0) were mixed with 1.5 ml of 10^{-3} M PMB dissolved in the same buffer. The cuvet was immediately placed into a thermostated cuvet holder at 30° and the A_{250} was recorded immediately and at fixed time intervals thereafter against a blank which contained PMB and buffer only. The net change in A_{250} was used to calculate the number of thiol residues titrated.

TABLE VI: The Effects of Some Compounds on Isocitrate Dehydrogenase Activity.[a]

Compound	Final Concn (M)	Initial Velocity, % of Control	
		A	B
None		100	100
2′-AMP	5×10^{-4}	90	85
3′-AMP	5×10^{-4}	87	92
5′-AMP	5×10^{-4}	95	96
3′,5′-Cyclic AMP	5×10^{-4}	94	100
ADP	5×10^{-4}	95	92
ATP	5×10^{-4}	92	100
DPN^+	5×10^{-4}	92	
cis-Oxaloacetate	5×10^{-4}	89	

[a] The initial velocity of each reaction was measured in the presence of the compounds indicated in the standard assay described in the text except as indicated. In expt A each assay was carried out with 0.3 μg of enzyme, specific activity 124 enzyme units/mg. In expt B the DL-isocitrate concentration was decreased to 4×10^{-4} M, and each assay was carried out with 0.09 μg of enzyme, specific activity 133 enzyme units/mg. The control contained no added reagents except those of the normal assay.

mately 30,000–40,000 (Sund, 1968). This dissociation of the native enzyme is sometimes related to the mode of regulation of the catalytic activity as exemplified by glutamate dehydrogenase (Frieden and Colman, 1967). Attempts were therefore made to determine if isocitrate dehydrogenase could be dissociated into subunits. If the enzyme could be shown to have subunits it might be possible to relate further information on the mechanism of regulation of its catalytic activity to other subunit-containing enzymes. Furthermore, detailed chemical studies on the primary structure of the enzyme would be simplified if the enzyme were composed of subunits. The molecular weight of the enzyme was therefore determined in the presence of 6 M guanidine hydrochloride and 0.01 M DTT by equilibrium centrifugation. The molecular weight thus determined was identical with that of the native enzyme, *viz.*, 80,000. The enzyme was not dissociated into subunits under these drastic denaturing conditions. Since the enzyme contains three thiol residues of differing reactivity per 80,000 g of protein, it might not be expected that the enzyme was composed of identical subunits if indeed subunits existed. Aspartate transcarbamylase has been effectively dissociated into subunits by treatment with PMB (Gerhart and Schachman, 1968). In an attempt to dissociate isocitric dehydrogenase with PMB, a sample of enzyme was titrated with PMB, excess reagent was removed, and the PMB-treated inactive enzyme was mixed with trace amounts of native enzyme. The mixture was subjected to get filtration on Sephadex G-100. There was no difference in the elution pattern of the PMB-treated enzyme and the trace quantities of native enzyme. The former was detected by absorbancy measurements at 280 nm and the latter by its catalytic activity. It might tentatively be concluded that the enzyme consists of a single polypeptide chain.

It has been reported by Colman (1968) that in pig heart isocitrate dehydrogenase the active site of the enzyme contains a methionine residue and perhaps two sulfhydryl groups (Colman, 1969). The data which we have reported indicate that the *A. vinelandii* enzyme contains a reactive sulfhydryl group which is essential for catalytic activity. The results which we have obtained indicate that variations in the active sites of the two enzymes from the different sources may indeed exist. The *A. vinelandii* enzyme is essentially devoid of activity when 1 mole of the thionitrobenzoic acid group is attached to 1 mole of enzyme. The catalytic activity is regenerated by treatment with a reducing agent such as DTT. Concomitant with this reactivation approximately 1 mole of chromophore is released per mole of enzyme. The specificity of the DTNB used to titrate the enzyme indicates that the group titrated is indeed a thiol. The rate of titration of the enzyme by DTNB is markedly reduced by addition of either TPN^+ or DL-isocitrate, which indicates that these substrates bind in the vicinity of the essential thiol. The binding of 1 mole of iodoacetate/mole of enzyme results in complete loss of catalytic activity. Finally the sulfhydryl reagents PMB, *N*-ethylmaleimide, and iodoacetamide all inhibit the enzyme. *N*-Ethylmaleimide when incubated at a concentration of 10^{-3} M with 3 enzyme units of purified enzyme at room temperature in 0.05 M potassium phosphate buffer (pH 8) resulted in approximately 50% loss of activity in 30 min. Iodoacetamide inactivates the enzyme at a very low rate. It was found that the apparent lack of effect of this reagent was due to its slow rate of reaction with the enzyme. After 24 hr at room temperature in 0.05 M potassium phosphate buffer (pH 8.0), 0.4 mole of [1-^{14}C]iodoacetamide was bound per mole of enzyme. The specific activity of the enzyme decreased from 130 to 93 enzyme units per mg as a result of this reaction. The evidence then indicates that one sulfhy-

dryl group in isocitrate dehydrogenase is at or close to the active site of the enzyme.

The three sulfhydryl groups of the enzyme exhibit different reactivities. The essential sulfhydryl group is the most reactive group. It reacts with a variety of reagents with an accompanying loss of catalytic activity. A second thiol residue exhibits little reactivity toward DTNB and IAA but is readily titrated by PMB. Complete loss of catalytic activity caused by titration with PMB is obtained only when 2 moles of this reagent react with 1 mole of enzyme. The reactivity of the second thiol group is perhaps increased by conformational changes in the protein molecule which are brought about when the first reactive thiol is titrated by the bulky PMB molecule. The binding of the first mercury therefore exposes the second thiol group which is very rapidly titrated. It is not uncommon for this reagent to enhance the reactivity of sulfhydryl groups attached to the same molecule which it attacks (Hellerman *et al.*, 1965). The third thiol group of the isocitrate dehydrogenase reacts slowly with PMB. Its reactivity to DTNB is enhanced by unfolding the protein molecule with guanidine hydrochloride. This third SH group is probably buried within the protein and is not readily accessible for reaction in the native enzyme.

There are a number of differences between the bacterial enzyme and the pig heart enzyme. The pig heart enzyme has a molecular weight of 58,000 (Colman, 1968) in contrast to 80,000 for the bacterial enzyme. The mammalian enzyme is reported (Colman, 1967) to have 12 cysteine residues/mole of enzyme, whereas the *A. vinelandii* enzyme contains only 3 sulfhydryl groups/mole of enzyme. There are additional differences in amino acid composition, notably a lower methionine content in the bacterial enzyme. The bacterial enzyme has a specific activity severalfold greater than the pig heart enzyme. The Michaelis constants for TPN^+ and D$_s$-isocitrate are 4.6×10^{-6} and 5.7×10^{-6} M, respectively, for the mammalian enzyme and 2.0×10^{-5} and 2.2×10^{-5} M, respectively, for the bacterial enzyme. At pH 9 the mammalian enzyme is positively charged and upon electrophoresis migrates to the cathode, but the bacterial enzyme at the same pH migrates to the anode.

In analogy with the DPN^+-specific isocitrate dehydrogenase from yeast (Hathaway and Atkinson, 1963) and *Neurospora* (Sanwal and Stachow, 1964) it was hoped that an allosteric modification of the enzyme by low molecular weight compounds such as the adenine nucleotides would be observed. Experiments with a variety of compounds at different concentrations of isocitrate have shown no evidence for such allosterism. The compounds had marginal effects on the catalytic activity of the enzyme. It is possible, however, that the appropriate conditions were not utilized to demonstrate allosteric effects.

The occurrence of the enzyme in such high concentrations in this microorganism is perhaps a reflection of its significance in the economy of the cell. It is of some interest that this bacterium like most other bacteria (Plaut, 1963) do not contain significant quantities of DPN^+-specific isocitrate dehydrogenase. In many of these bacteria high levels of TPN^+–isocitrate dehydrogenase are accompanied by high levels of pyridine nucleotide transhydrogenase although there are exceptions (Ragland *et al.*, 1966). In *Pseudomonas fluorescens* (Kaplan *et al.*, 1953) and *A. vinelandii* (Kaplan *et al.*, 1953; A. E. Chung, unpublished observations, 1968) the pyridine nucleotide transhydrogenases show a remarkable preference for transferring reducing equivalents from TPNH to DPN^+; only under special conditions do these enzymes transfer reducing equivalents from DPNH to TPN^+. It is not known with any certainty what the function of the transhydrogenases are but in *A. vinelandii* it is tempting to suggest that the combination of TPN^+–isocitrate dehydrogenase and pyridine nucleotide transhydrogenase offers a very powerful pathway to generate DPNH for energy production and for providing the reducing atmosphere for nitrogen fixation.

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The Mechanistic Significance of Phosphate Labeling of Alkaline Phosphatase*

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ABSTRACT: Alkaline phosphatase reacts with orthophosphate at pH <8 to form a thermodynamically stable phosphoprotein. In order to study this phosphoprotein, a rapid quenching technique in conjunction with scintillation counting was developed which allowed for an accurate determination of amount of phosphoprotein formed. The reaction was stopped in 6 M perchloric acid by forcing 0.5 ml/sec of the solution through a 0.020-i.d. capillary tip into the quenching solution by means of a motor-driven syringe. The covalent labeling was measured from pH 8.0 to 5.0 and at different concentrations of phosphate. In this way the equilibrium constants for the dissociation of the enzyme–phosphate addition complex at 26° (Michaelis complex) were found to be 4.1×10^{-6} at pH 7.0, 6.7×10^{-6} at pH 6.0, and 5.4×10^{-5} at pH 5.5, while the equilibrium constants for the hydrolysis of the covalent phosphoprotein were found to be 1.4×10^{-4} at pH 7.0, 6.0×10^{-6} at pH 6.0, and 1.2×10^{-5} at pH 5.5. Measurements were also made at 3°. These constants are in good agreement with the values of these quantities for the phosphoryl-enzyme intermediate determined previously by kinetic methods. Similarly the calculated kinetic inhibition constants for phosphate at 26°, 3.8×10^{-6} at pH 7.0, 3.1×10^{-6} at pH 6.0, and 1.0×10^{-5} at pH 5.5, agreed with the experimental values. Thus the properties of the kinetically deduced phosphoryl-enzyme intermediate are the same as the properties of the phosphoprotein obtained in labeling experiments so that there is good reason to conclude that the two are the same. The phosphoprotein is stable relative to free enzyme and inorganic phosphate at all pH's studied, yet very little is formed at pH 7.0 and 8.0. The reason for this seeming anomaly is that the addition complex between enzyme and phosphate is even more stable at these pH's. As the pH is lowered, the relationship changes so that at pH 5.5 the covalent phosphoryl-enzyme is much more stable than the enzyme–phosphate complex and labeling is extensive.

Several workers have confirmed and extended the important observation of Engstrom and Agren (1958) that a phosphoprotein is formed when ^{32}P-labeled inorganic phosphate is added to a solution of alkaline phosphatase (Engstrom, 1961, 1962a,b, 1964; Schwartz, 1963; Schwartz and Lipmann, 1961; Milstein, 1964; Pigretti and Milstein, 1965). The phosphate is covalently linked to serine (Schwartz and Lipmann, 1961).

It is not suprising that protein should be phosphorylated in this procedure because alkaline phosphatase in its role as a nonspecific catalyst must catalyze the formation of phosphate esters as well as their hydrolysis and indeed alkaline phosphatase has been used for just this purpose. It is therefore to be expected that all groups that can serve as phosphate acceptors will be phosphorylated to an extent depending upon the thermodynamic stabilities of the resulting phosphate derivatives. In the present instance we have the surprising result that the phosphoprotein that is formed is about one million times more stable than ordinary phosphate esters (Wilson and Dyan, 1965).

For just the reasons discussed above, the labeling of protein in alkaline phosphatase preparations is a matter of thermodynamics and not of mechanism and therefore does not prove that a phosphoryl-enzyme is formed as an intermediate during the catalyzed hydrolysis of phosphate esters. However, other observations of a kinetic nature either suggest or indicate that a phosphoryl-enzyme intermediate occurs (Aldridge *et al.*, 1964; Fernley and Walker, 1966; Fife, 1967; Williams, 1966;

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