Activation and Inhibition of DPN-linked Isocitrate Dehydrogenase of Heart by Certain Nucleotides*

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DPN-linked isocitrate dehydrogenase has been purified from mitochondrial acetone powder of bovine heart. The purified protein exhibits a major component having a sedimentation constant of 10.3 S in the analytical ultracentrifuge, with a possible molecular weight in the range $3–4 \times 10^5$. ADP has been found to affect the enzyme in several ways. The nucleotide stabilizes the enzyme under conditions of low ionic strength. ADP also enhances the activity of the enzyme, and this effect has been found to be due chiefly to a marked decrease in the K_m for isocitrate. Thus, at low isocitrate concentrations the enzyme is virtually dependent on ADP for activity. This effect of ADP is highly specific, since, of a large number of nucleotides tested, only ADP and dADP are stimulatory. In the presence of low concentrations of isocitrate, the *p*H optimum is displaced from *p*H 6.7 in the absence of ADP to about *p*H 7.2 in the presence of ADP. A similar shift of the *p*H optimum is obtained by increasing the substrate concentration in the absence of ADP. The enzyme is inhibited by DPNH, ATP, and ADPR; the inhibition is competitive with DPN^+. TPNH potentiates the DPNH inhibition of the DPN-linked isocitrate dehydrogenase. On the other hand, the initial reaction rate of the TPN-linked isocitrate dehydrogenase system is not affected by ADP, ATP, DPN^+, or DPNH. In the presence of DPN-linked isocitrate dehydrogenase, the fluorescence emission spectrum of DPNH is altered, suggesting the formation of a DPNH-enzyme complex. The significance of the inhibition by ATP and DPNH, as well as the ADP stimulation, is discussed in terms of a possible mechanism for regulation of cardiac mitochondrial oxidation.

It has been shown (Plaut and Sung, 1954) that a number of animal tissues contain a DPN-linked isocitrate dehydrogenase catalyzing the reaction

threo-D$_s$-isocitrate[1] + DPN^+ ⟶

α-ketoglutarate + CO_2 + DPNH + H^+

A preliminary communication (Chen and Plaut, 1962) reported that enzyme from bovine heart was markedly stimulated by ADP and that this nucleotide markedly decreased the apparent Michaelis constant K_m for isocitrate. The discovery of the activating effect of ADP on this enzyme occurred during the course of a systematic survey of the effect of various nucleotides. This study was undertaken for several reasons. First, Kornberg and Pricer (1951), who initially described a DPN-linked isocitrate dehydrogenase, found that the yeast enzyme was almost fully dependent on minute quantities of 5′-AMP, although ADP in larger amounts could also activate the enzyme. In *Aspergillus niger*, DPN-linked isocitrate dehydrogenase has been reported to be activated by 5′-AMP and by inorganic phosphate (Ramakrishnan and Martin, 1955). Although 5′-AMP had no such effect on the mammalian DPN-specific isocitrate dehydrogenase (Plaut and Sung, 1954), it remained possible that the enzyme might be activated by other nucleotides. Second, the DPN-linked enzyme of cardiac muscle had been observed to be inhibited by ATP (Plaut and Sung, 1954). Also the rate of isocitrate oxidation by the DPN-linked enzyme decreased markedly with time, and it was found that this effect was due to product inhibition by DPNH. The inhibition by DPNH and ATP was counteracted competitively by DPN^+ (Chen and Plaut, 1962). The nature of inhibition by these and other nucleotides has been examined and the results are reported in detail in this communication.

Studies of the kinetic properties of DPN-specific isocitrate dehydrogenase have been hampered previously by the unavailability of adequate amounts of highly purified enzyme. The enzyme is unstable and is generally recovered from animal tissues in much lower amounts than the well-studied TPN-linked isocitrate dehydrogenase (Plaut and Sung, 1954). In the present work, improved methods have been developed to stabilize and to purify the enzyme. The methods for stabilizing the enzyme have been useful in the studies on the nature of the hydrogen transfer catalyzed by this enzyme (Chen and Plaut, 1963) and in the present investigation of the effect of various nucleotides on the rate of reaction.

MATERIALS AND METHODS

The following compounds[2] were obtained from the Sigma Chemical Co., St. Louis, Mo.: DPN^+, the α isomer of DPN^+ (α-DPN^+), DPNH, TPN^+, TPNH, 5′-AMP, ADP, dADP, ATP, ADPR, 3′,5′-cyclic-AMP, IDP, ITP, GDP, GTP, UMP, UDP, CDP, CTP, riboflavin, riboflavin 5′-phosphate, and FAD. Adenine, adenosine, 2′-AMP, and 3′-AMP were obtained from Schwarz BioResearch, Inc., Orangeburg, N.Y. dGMP and dAMP were obtained from California Corporation for Biochemical Research, Los Angeles, Cal. The following millimolar extinction coefficients (liter millimole^{-1}) were assumed for assay purposes

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[1] For nomenclature, see Vickery (1962)

[2] The abbreviations used are those approved by the *Journal of Biological Chemistry*. In addition, DEAE-cellulose is diethylaminoethylcellulose, ADPR is adenosine diphosphate ribose. An example of the abbreviations used for pyridine nucleotide analogs is acetylpyridine-DPN^+, the 3-acetylpyridine analog of DPN^+.

(Dawson *et al.*, 1959): 15.4 for adenine-containing nucleotides at 259 mμ; 12.2 for hypoxanthine-containing nucleotides at 262 mμ; 13.7 for guanine-containing nucleotides at 252 mμ; and 13.0 for cytosine-containing nucleotides at 280 mμ. NMN^+, acetylpyridine-DPN^+ deamino-DPN^+, thionicotinamide-DPN^+, and pyridinealdehyde-DPN^+ were obtained from Pabst Laboratories, Milwaukee. The extinction coefficients and absorption maxima used to determine the corresponding reduced nucleotides were those reported by Siegel *et al.* (1959). The reduced forms of the DPN analogs were prepared by dithionite reduction (Conn *et al.*, 1952).

threo-D_sL_s-Isocitric acid lactone from the California Corp. for Biochemical Research was hydrolyzed by heating at 95° at *p*H 10 for 20 minutes and was neutralized with HCl before use. Enzymic assay with TPN-linked isocitrate dehydrogenase (Plaut, 1962) indicated that the isocitrate was 99–100% pure. Although *threo*-D_sL_s-isocitrate was used in all experiments, concentrations are expressed in terms of the *threo*-D_s isomer. The barium salt of glucose 6-phosphate was obtained from Sigma Chemical Co. and converted to a solution of the potassium salt before use. α-Ketoglutaric acid was obtained from the Aldrich Chemical Co. and purified by recrystallization from ether and benzene. Glucose 6-phosphate dehydrogenase, Type V, and crystalline bovine liver L-glutamate dehydrogenase (Type I, ammonium sulfate suspension) were purchased from the Sigma Chemical Co.

Ammonium sulfate solutions used in the purification procedure were adjusted to *p*H 7.0 by addition of concentrated ammonium hydroxide. Per cent saturation was calculated on the basis of solubility at 25° although enzyme fractionation was done at 2–5°. The actual ammonium ion content of the solutions was determined by a modification of the Nessler method (Johnson, 1949).

DEAE-cellulose from the Brown Company, Berlin, N.H., 0.9 meq/g, was washed by the procedure of Kaziro *et al.* (1961), equilibrated with 0.005 M potassium phosphate buffer at *p*H 7.2, and packed into 2.2 × 12-cm columns. Hydroxylapatite was prepared as described by Levin (1962). Columns of hydroxylapatite (2.2 × 12 cm) which were allowed to pack by gravity had a flow rate of 2.2 ml/minute with a pressure of 20 cm of water. Sephadex G-200 was obtained from Pharmacia Fine Chemicals, Inc., Rochester, Minn.

Assay of Enzymic Activity.—DPN-linked isocitrate dehydrogenase activity was measured by following the production of DPNH as reflected in the increase in optical density at 340 mμ. Measurements of initial reaction rates were made with a Zeiss PMQ II spectrophotometer fitted with a Varicord linear recorder (Photovolt Corp., N.Y.). The temperature was maintained at 25° by means of a thermostated cuvet compartment. The assay procedure as previously described (Plaut and Sung, 1954; 1955) has been altered with respect to the reaction mixture components, which now consist of the following: Tris acetate buffer, *p*H 7.2, 100 μmoles; $MnCl_2$, 4.0 μmoles; ADP, 2.0 μmoles; DPN^+, 1.0 μmole; *threo*-D_sL_s-isocitrate, 16.0 μmoles; water; and enzyme, in a final volume of 3.0 ml. Deviations from these conditions of incubation are indicated in the text, tables, and figures. One unit of enzyme is defined as that amount which causes a change of 0.01 in optical density at 340 mμ per minute at 25° in a cell of 1.0 cm path length under these conditions.

Chromatography of Nucleotides.—The method of Krebs and Hems (1953) was employed for the detection of adenosine triphosphatase and adenylate kinase in enzyme preparations at varying stages of purification. Ascending paper chromatography was carried out on Whatman No. 3 MM paper with an isobutyric acid–1 N ammonia–0.1 M EDTA (100:60:1.6) system. The solvent system (Hanes and Isherwood, 1949) isopropyl ether–90% (w/v) formic acid (90:6.0), was used in addition for the separation of products in experiments where ADP^{32} was present.[3]

Other Determinations.—Glutamate was determined by the method of Moore and Stein (1954). Protein was determined by the method of Warburg and Christian (1941). Fluorescence spectra were measured with a Farrand spectrophotofluorimeter fitted with a General Electric Co. Type AH-4 mercury lamp.

Results

Preparation of DPN-linked Isocitrate Dehydrogenase from Bovine Heart[4]

Preparation of Bovine Heart Acetone Powder.—The procedure of Plaut and Sung (1954) was employed, except that the acidification of the supernatant fluid after low speed centrifugation of a sucrose-phosphate homogenate of bovine heart was extended to *p*H 5.5 instead of *p*H 5.8–5.9. This generally resulted in greater recovery of enzymic activity but lower specific activity in the initial phosphate buffer extract (step 1, below). 232 g of acetone powder was obtained from 30 kg of bovine heart.

All operations of the purification procedure were performed at 2–5° unless otherwise specified.

1. Extraction. Acetone powder (232 g) was homogenized in a Waring Blendor with 4000 ml of 0.10 M potassium phosphate buffer, *p*H 7.2. The homogenate was centrifuged at 1000 × *g*, for 20 minutes and the residue was homogenized with an additional 1600 ml of buffer. After centrifugation, the supernatant solutions were combined.

2. Ammonium sulfate fractionation and heat step. The extract was treated with solid ammonium sulfate while the reaction was maintained at *p*H 7.0–7.4 by addition of solid Na_2CO_3 (approximately 0.1% of the weight of the ammonium sulfate added). The material precipitating between 30% and 50% saturation was mixed with 500 ml of 30% saturated ammonium sulfate solution in a Potter-Elvehjem homogenizer. The suspension was heated in a water bath, and the temperature was maintained at 50° for 15 minutes. The suspension was then rapidly cooled in an ice bath and centrifuged. The sediment was discarded.

It was noted that a reproducible increase in activity of about 30% resulted from heating the protein in 30% saturated ammonium sulfate solution in this step.

3. Ammonium sulfate fractionation. Saturated ammonium sulfate solution was added to the supernatant fluid from step 2. The material precipitating between 35% and 45% saturation was collected by centrifugation and stored after suspending in 60 ml of 30% saturated ammonium sulfate solution.

4. Dialysis. Saturated ammonium sulfate (8.0 ml) was added to a 20-ml aliquot of the enzyme suspension from step 3. The precipitate was collected by centrifugation and dissolved in 27 ml of 0.005 M potassium phosphate buffer, *p*H 7.2, containing 0.001 M ATP, and was dialyzed for two hours with high speed stirring against

[3] We wish to thank Dr. M. Chiga for a gift of ADP^{32} labeled in the terminal phosphate group, prepared as previously described (Chiga and Plaut, 1959).

[4] The authors are grateful to Dr. Günther Siebert for helpful discussions and collaboration in the early phases of the enzyme purification.

TABLE I

PURIFICATION OF DPN-LINKED ISOCITRATE DEHYDROGENASE FROM BOVINE HEART

Step	Volume (ml)	Activity (units/ml)	Total Activity (units)	Activity Yield (%)	Total Protein (mg)	Specific Activity (units/mg)	Purification (fold)
(1) Crude extract	5,170	44	217,000	100	37,000	6.2	1
(2) Ammonium sulfate and heat treatment	525	528	277,200	128	3,680	75	12.5
(3) Ammonium sulfate fractionation	62	4,500	279,000	129	2,090	134	22
(4) Dialysis[a]	37	2,280	84,800	100	405	200	32
(5) DEAE-cellulose chromatography[a]	14	3,200	45,080	53	42	1,070	173
(6) Hydroxylapatite chromatography[a]	1.7	19,500	33,200	39	7.4	4,500	727

[a] One third of the protein from step 3 was purified in steps 4 to 6. The activity yield has been adjusted for the change in amounts.

2000 ml of 0.005 M potassium phosphate buffer, *p*H 7.2. The cloudy precipitate was discarded. The ammonium ion content of the enzyme solution was usually found to be 0.03–0.04 M at this point. By slow addition of buffer, the enzyme solution was diluted until the ammonium ion content was 0.02 M.

5. Chromatography on DEAE-cellulose. Portions of the enzyme solution from step 4 were placed on four DEAE-cellulose columns previously equilibrated against 0.005 M potassium phosphate buffer, *p*H 7.2. About 100 mg of protein was placed on each 2.2 × 12-cm column. A linear gradient was applied to the column to elute the protein by means of a mixing chamber containing 150 ml of 0.005 M potassium phosphate buffer, *p*H 7.2, and a reservoir containing 150 ml of buffer plus 0.2 M NaCl. A uniform flow rate of 2.2 ml/minute was maintained with a constant-volume delivery pump (Mini-Pump, Milton Roy Co., Philadelphia). Fractions (15.0 ml) were collected and the tubes with the highest specific activity were pooled. An equal volume of saturated ammonium sulfate solution was added to the pooled fractions to precipitate the protein. After centrifugation, the enzyme was stored as the precipitate beneath the supernatant solution. Prior to the next step, the protein was dialyzed against buffer as in step 4, but without ATP.

6. Hydroxylapatite chromatography. The enzyme was applied to a 2.2 × 12-cm hydroxylapatite column previously equilibrated against 0.005 M potassium phosphate buffer, *p*H 7.2. It was eluted from the column with a linear gradient system consisting of 150 ml of 0.005 M potassium phosphate at *p*H 7.2 in the mixing chamber and 150 ml of 0.2 M potassium phosphate buffer, *p*H 7.2, in the reservoir. A flow rate of 1.5 ml/minute was maintained and 5.0-ml fractions were collected. The fractions with the highest specific activities (3410 to 5520 units per mg of protein) were pooled and the protein was precipitated by adding saturated ammonium sulfate to give a 60% saturated solution. The protein was centrifuged down and stored in the centrifuge tube beneath the supernatant fluid. The protein was dissolved and dialyzed for ultracentrifugal analysis as described below.

A summary of the purification procedure is given in Table I.

Properties of the Enzyme

Stability.—Initial attempts at purification of large amounts of enzyme were unsuccessful because of the marked instability of the enzyme at low ionic strength. It was routinely found that over 50% of the activity was lost in 24 hours at 2°, when the ionic strength of the medium was about 0.1. However, in solutions containing 5×10^{-3} M ADP, 90% of the activity remained after 4 days, even at an ionic strength of 0.1. In crude preparations, ATP stabilized the enzyme about as well as ADP; but ATP was ineffective in highly purified preparations, whereas ADP continued to stabilize. The stabilization of enzyme activity by ATP in the crude fractions is probably attributable to the conversion of the nucleoside triphosphate to ADP by the action of adenosine triphosphatase and adenylate kinase. These enzymes have been shown to be present in preparations prior to the DEAE-cellulose chromatography step (step 5, Table I).

Because of the relatively low cost of ATP, this nucleotide was used in the dialysis (step 4, Table I) preceding DEAE-cellulose chromatography to prevent loss of enzyme activity.

It was found that the enzyme was quite stable in 30% saturated ammonium sulfate solutions, and could be stored for at least 4 weeks at 2° in this manner without significant loss of activity. However, the enzyme appeared less stable in 40% or more saturated ammonium sulfate solutions.

Ultracentrifugation of the Purified Enzyme.[5]—The protein of step 6 (Table I) of the purification procedure was taken up in 1.0 ml of 1×10^{-4} M ADP and dialyzed for 60 minutes against 1000 ml of 0.10 M potassium phosphate buffer, *p*H 7.2, containing 1×10^{-5} M ADP. When centrifuged at 59,780 rpm in a Spinco Model E ultracentrifuge at 20°, the sample showed one major (about 85%) and one minor (about 15%) component sedimenting at rates of 10.3 S and 4.6 S, respectively. After centrifugation, the top third of the cell contents containing the component sedimenting at 4.6 S was drawn off with a syringe. Although much mixing occurred during this procedure, the fluid at the bottom of the cell had 83% of the enzymic activity and a specific activity 3 times higher than the fluid removed. A separate sample of enzyme with a specific activity of 3200 was prepared in a slightly different manner and contained, on ultracentrifugation, a major component (10.3 S) and a minor component (6.2 S). In the preparation of this second sample, DEAE-cellulose chromatography was replaced by another hydroxylapatite-chromatography step in which adsorbed enzyme was washed on the column with 10% saturated ammonium sulfate solution and eluted with 20% saturated ammonium sulfate solution. Because both enzyme preparations contained the 10.3 S component but differed as to the minor component, it is likely that the DPN-linked isocitrate dehydrogenase is associated with the large molecular weight component (10.3 S). This possibility was enhanced by the finding, in separate ex-

[5] We wish to thank Mr. D. M. Brown for performing the ultracentrifuge measurements.

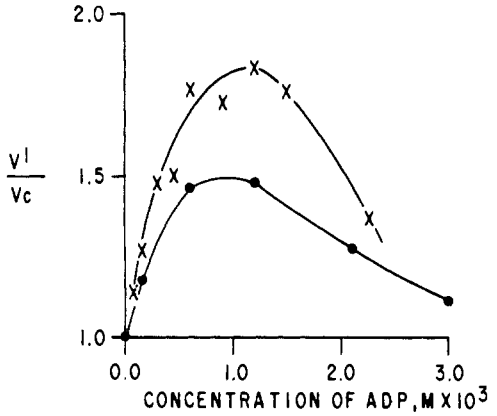

FIG. 1.—The effect of ADP on initial reaction rates as a function of ADP concentration. All measurements were taken at 25°. Each cuvet contained in a final volume of 3.0 ml: 100 μmoles of Tris-acetate buffer, *p*H 7.2; 4.0 μmoles of $MnCl_2$; 1.0 μmole of DPN^+; and 10 μg of enzyme (specific activity, 1001 units per mg). The concentrations of ADP and isocitrate were varied. ×—×, 6.7 × 10^{-4} M *threo*-D_s-isocitrate; ●—●, 2.7 × 10^{-3} M *threo*-D_s-isocitrate. *V'* is the rate in the presence of ADP and *Vc* is the rate in the absence of ADP. The reactions were initiated by the addition of enzyme.

periments, that the passage of the enzyme through a column of Sephadex G-200 was essentially unretarded by a molecular sieve capable of holding substances up to a molecular weight of 200,000.

Effects of Various Nucleotides

Stimulation of Activity by ADP.—Although 5'-AMP had little or no effect on the enzyme, and ATP was inhibitory towards DPN-linked isocitrate dehydrogenase from bovine heart (Plaut and Sung, 1954), ADP was found to stimulate the reaction in concentrations as low as 6.7 × 10^{-5} M. The greatest amount of stimulation occurred at an ADP concentration of about 1 × 10^{-3} M; however, it is apparent from the results shown in Figure 1 that *V'/Vc*, the ratio of the rate in the presence of ADP (*V'*) to the rate without ADP (*Vc*), is greater at lower isocitrate concentrations. It thus seemed possible that ADP stimulated the enzyme by increasing the apparent affinity of the enzyme for isocitrate. Therefore the effect of variation of isocitrate concentration on the rate of the reaction was measured in the presence and absence of ADP. The results of such an experiment are shown in Figure 2. It is evident that the stimulation by ADP can be attributed mainly to a marked decrease in the apparent Michaelis constant K_m for isocitrate. The K_m values have been calculated from the curves shown in Figure 2 by the method of Lineweaver and Burk (1934) and are summarized in Table II. Thus at *p*H 7.2, K_m for isocitrate is about 10-fold greater in the absence of ADP (K_m, 1.5 × 10^{-3} M) than in the presence of the nucleotide (K_m, 1.4 × 10^{-4} M). This disparity in K_m values is reflected by the markedly different slopes of the curves obtained for the experiments with and without ADP (Fig. 2A). At a lower *p*H, the effect of ADP is still present but it is less marked. The corresponding curves for *p*H 6.5 are given in Figure 2B, and the calculated K_m values with and without ADP are 1.0 × 10^{-4} M and 3.6 × 10^{-4} M, respectively. At both *p*H 7.2 and *p*H 6.5 the lines converge at the ordinate (Fig. 2, A and B) so that V_{max} is about the same both with and without ADP. It is evident that the affinity of the enzyme for isocitrate is much less at *p*H 7.2 than at *p*H 6.5 (Table II). As a result, at *p*H 7.2 with lower concentrations of isocitrate, the rate is relatively slow.

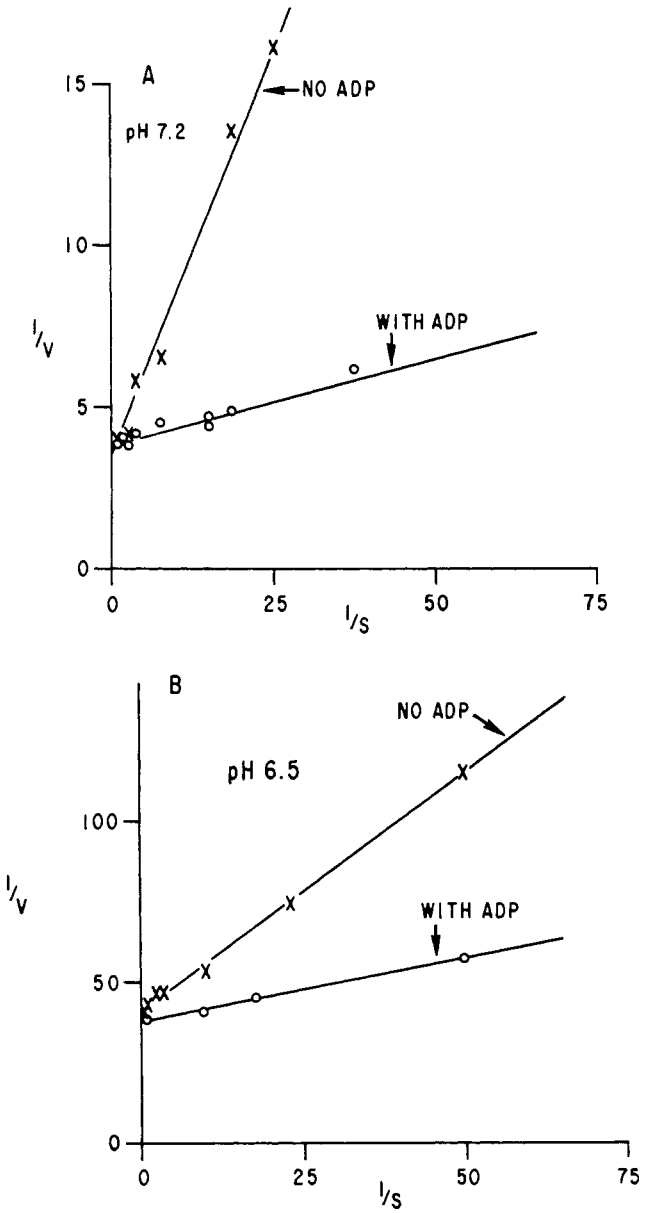

FIG. 2.—Velocity as a function of isocitrate concentration in the presence and absence of ADP. The reciprocal of velocity (*V*, change in optical density at 340 mμ/minute) is plotted against the reciprocal of isocitrate concentration (*S*, concentration of *threo*-D_s-isocitrate, M × 10^2) for experiments done at *p*H 7.2 (A) and at *p*H 6.5 (B). The conditions of incubation were as described under "Assay of Enzymic Activity," except that the concentration of isocitrate was varied and 6.7 × 10^{-4} M ADP was either present (○—○) or absent (×—×) from the reaction mixtures. In addition, in the experiments of Figure 2B, the usual Tris-acetate buffer was replaced by potassium cacodylate buffer, *p*H 6.5. In the experiments of Figure 2A, 25 μg of protein (specific activity, 1001) was used per cuvet; about 22 μg of protein (specific activity, 200) per cuvet was present in the experiment represented by Figure 2B.

Under these conditions the stimulatory effect of ADP becomes more dramatically apparent (Fig. 2A). Thus, when *threo*-D_s-isocitrate concentration is 1.0 × 10^{-3} M at *p*H 7.2 it can be calculated that the ADP-stimulated reaction rate is 150% of the rate without ADP. At still lower concentrations of isocitrate the enzyme shows a virtually complete dependence on ADP for activity. For example, at 1 × 10^{-4} M *threo*-D_s-isocitrate the velocity of the reaction without ADP is only 1–2% of the rate in the presence of ADP.

TABLE II

K_m FOR ISOCITRATE WITH AND WITHOUT ADP[a]

Conditions	K_m
pH 6.5, no ADP	3.6×10^{-4} M
pH 6.5, 6.7×10^{-4} M ADP	1.0×10^{-4} M
pH 7.2, no ADP	1.5×10^{-3} M[b]
pH 7.2, 6.7×10^{-4} M ADP	1.4×10^{-4} M[b]

[a] These values are calculated from the data of Fig. 2.
[b] The present K_m values were obtained with a more highly purified preparation of enzyme than used in an earlier study (Chen and Plaut, 1962) which indicated that ADP reduced the K_m at this pH by at least 5-fold.

A further effect of ADP is to cause an apparent shift of the pH optimum. In the original study on this enzyme (Plaut and Sung, 1954) it was reported that the optimal activity occurred at pH 6.5 in cacodylate buffer, when the *threo*-D_sL_s-isocitrate concentration was 2.7×10^{-3} M (equivalent to 1.3×10^{-3} M of the *threo*-D_s isomer). Figure 3 shows the reaction rate as a function of the pH for high and low concentrations of isocitrate as determined in imidazole and Tris buffers. Figure 3 illustrates the following points: (a) When isocitrate concentration is relatively low (1.3×10^{-3} M), the maximal activity is attained at pH 6.7 in the absence of ADP, but the pH optimum is shifted to pH 7.2 in the presence of 6.7×10^{-4} M ADP (upper and lower curves). (b) A similar shift in pH optimum is obtained in the absence of ADP by increasing the isocitrate concentration 10-fold (middle curve). (c) When isocitrate concentration is low and ADP is absent, there is only a rather narrow range of pH in the region of 6.5–6.9 where much activity is obtained. This correlates well with the data of Table II, which indicates that the apparent affinity of the enzyme for isocitrate is greater at pH 6.5 than at pH 7.2, provided ADP is absent. (d) The velocity-vs.-pH curves are broader, with maxima at pH 7.2, when ADP is present, or when ADP is absent but isocitrate concentration is high.

As an explanation for stimulation of the enzyme by ADP, two possibilities were considered here: (1) ADP is a participant in the over-all reaction in a process in which the nucleotide is consumed in the reaction yielding 5′-AMP and orthophosphate as products. (2) ADP reacts with the enzyme system, leading to the formation of an activated enzyme complex.

The possibility that ADP participates in stoichiometric amounts in the DPN-linked isocitrate dehydrogenase reaction was tested by using ADP^{32} labeled in the terminal phosphate group. In a final volume of 0.3 ml were placed 10.0 μmoles of Tris buffer, pH 7.2; 0.4 μmole of $MnSO_4$; 1.6 μmoles of *threo*-D_sL_s-isocitrate, 0.1 μmole of DPN^+; 0.2 μmole of ADP^{32}; and 3 units of enzyme, specific activity 725 (4.1 μg total). After 35 minutes at 23°, the reaction mixture was deproteinized with 10% trichloroacetic acid and an aliquot of the supernatant fluid was chromatographed on paper in the isopropyl ether–formic acid system of Hanes and Isherwood (1949), and in the isobutyric acid–ammonia–ethylenediaminetetraacetate system (Krebs and Hems, 1953). All the radioactivity was found in the spot corresponding to ADP, except for a small amount in ATP. No radioactivity was found in the area corresponding to orthophosphate. Since the radioactive ATP spot occurred to the same extent in a control reaction mixture not containing isocitrate, it is probable that the enzyme preparation contained a slight amount of adenylate kinase. Thus, ADP seems to stimulate in *catalytic* rather than in stoichiometric amounts.

If ADP stimulated by forming an activated enzyme complex, it seemed possible that a lag period might be detectable between the time of addition of ADP and the attainment of full activation. Kearney (1957) has shown that the activation of succinate dehydrogenase by malonate, succinate, and phosphate is a relatively slow process involving large energy changes and probably is accompanied by changes in protein configuration. Therefore, attempts were made to detect a lag period in the activation of DPN-linked isocitrate dehydrogenase by ADP. The experiments involved rapid addition of ADP to the contents of cells in a Cary recording spectrophotometer at 25°. No lag period was observed although any delay longer than three seconds would have been detected.

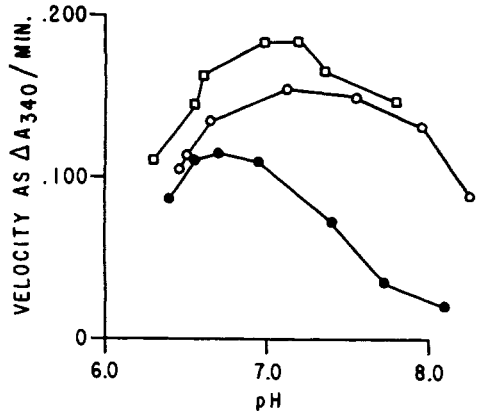

FIG. 3.—Effect of isocitrate and ADP on pH optimum. All reactions were run at 25° and initiated by addition of enzyme. Each reaction mixture of 3.0 ml contained 100 μmoles of imidazole chloride buffer (below pH 7.0) or Tris acetate buffer (pH 7.0 or above), 4.0 μmoles of $MnCl_2$, 1.0 μmole of DPN^+, 25 μg of protein (specific activity, 400). Isocitrate and ADP varied as follows: □—□, 1.3×10^{-3} M *threo*-D_s-isocitrate and 6.67×10^{-4} M ADP; ○—○, 1.3×10^{-2} M *threo*-D_s-isocitrate and no ADP; ●—●, 1.3×10^{-3} M *threo*-D_s-isocitrate and no ADP.

The effect of ADP is not exerted at the site of the coenzyme since the K_m of DPN^+ was not affected significantly at pH 7.2, being around 8×10^{-4} M both with and without ADP.

The activation of ADP seems to be specific, since the following compounds had no such effect when tested in the concentration range of 1×10^{-3} M to 1×10^{-4} M: adenine, adenosine, 2′-AMP, 3′-AMP, 5′-AMP, 3′, 5′-cyclic-AMP, dGMP, dAMP, UMP, ADPR, IDP, GDP, UDP, CDP, ITP, GTP, ATP, CTP, TPN^+, riboflavin, riboflavin 5′-phosphate, and FAD. However, dADP was about 10% more stimulatory than ADP at the same concentration. The fact that only ADP and dADP stimulate the enzyme indicates that a nonspecific effect, such as metal chelation, is unlikely as the basis of the activation. No effect of ADP on TPN-linked isocitrate dehydrogenase could be detected.

Inhibition by Nucleotides.—The rate of DPNH formation in the DPN-linked isocitrate dehydrogenase reaction diminished with time due to product inhibition by the DPNH formed (Chen and Plaut, 1962). This inhibition was competitive with DPN^+ as shown by the Lineweaver-Burk plots represented in Figure 4B. The inhibition constant of DPNH, K_I, was calculated to be 3.9×10^{-5} M while K_m for DPN^+ was 7.8×10^{-5} M. The inhibition by DPNH was not competitively reversed by isocitrate. It was interesting that DPNH was inhibitory, since it has not been possible to demonstrate the reversibility of the DPN-linked isocitrate dehydrogenase reaction catalyzed by the enzyme from heart (Plaut and Sung, 1954). The magnitudes

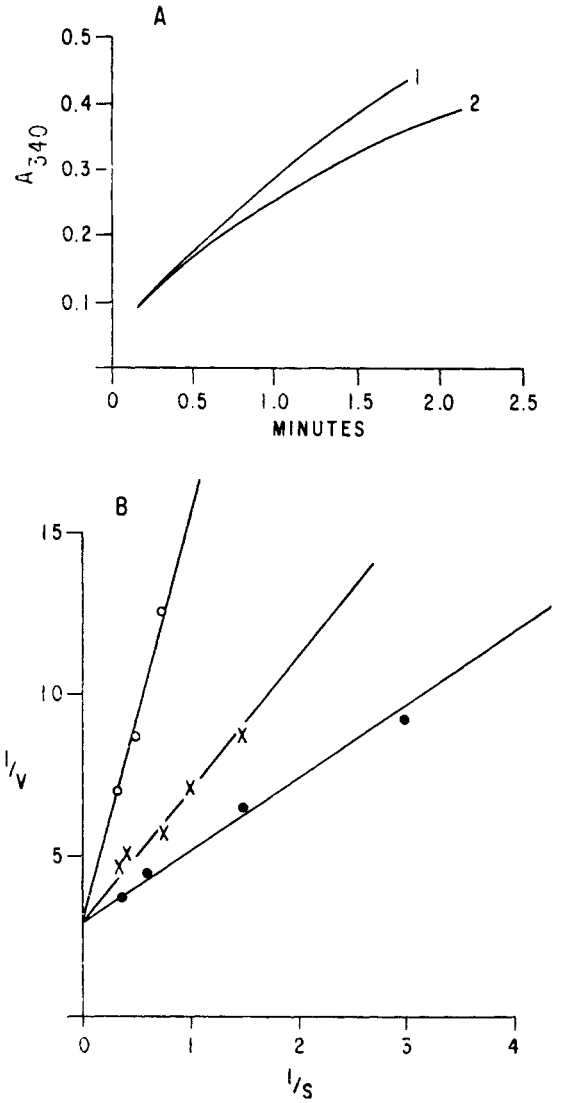

FIG. 4.—Inhibition by DPNH and TPNH. (A) Continuous recordings showing the optical density at 340 mμ as a function of time. The cuvets contained the usual assay system in a volume of 3.0 ml., including, curve 1, 3.3×10^{-4} M DPN^+; curve 2, 3.3×10^{-4} M DPN^+ and 3.3×10^{-4} M TPNH; 17 μg of protein per cuvet (specific activity, 3200). (B) Plot of the reciprocal of velocity against the reciprocal of DPN^+ concentration. The usual assay system was used for each reaction except that the DPN^+ content was varied and inhibitor was added in the experiments as indicated below. ●—●, no reduced pyridine nucleotides were present initially; ×—×, 3.3×10^{-5} M DPNH present initially; ○—○, 3.3×10^{-5} M DPNH and 3.3×10^{-5} M TPNH were present initially. *V*, velocity expressed as change in optical density at 340 mμ/minute; *S*, concentration of DPN^+, M × 10^3. Enzyme, same as in part A.

of the K_m for DPN^+ and the K_I for DPNH suggest that the enzyme has greater affinity for DPNH. The other products of isocitrate oxidation, CO_2 and α-ketoglutarate, were found to exert no effect on initial reaction rate.

The finding that DPNH was inhibitory was confirmed by showing that substrate utilization remained linear when DPNH was removed as fast as formed. Thus the rate of DPNH formation in the isocitrate dehydrogenase reaction declined with time of incubation (Fig. 5A and curve 1 of Fig. 5B). If under these conditions an amount of glutamate dehydrogenase adequate to cause instantaneous removal of the DPNH formed (Fig. 5A) was present in the incubation mixture prior to starting the reaction with isocitrate dehydrogenase, the rate of glutamate formation, a measure of isocitrate oxidation in the coupled reaction,

threo-D_s-isocitrate + DPN^+ ⟶ α-ketoglutarate + CO_2 + DPNH + H^+

α-ketoglutarate + DPNH + H^+ + NH_3 ⟶ glutamate + DPN^+

Sum: *threo*-D_s-isocitrate + NH_3 ⟶ glutamate + CO_2

was linear (curve 2 of Fig. 5B) because DPNH accumulation had been prevented.

TPNH was found to have an unusual effect on DPN-linked isocitrate dehydrogenase. This nucleotide showed essentially no inhibition of the *initial* reaction rate, but altered the velocity of isocitrate oxidation with time. Typical curves representing reactions with and without TPNH are shown in Figure 4A. TPNH apparently potentiates the inhibitory effect of DPNH, so that the usual decrease in velocity with time due to product inhibition by DPNH (Fig. 4A, curve 1) becomes more marked in the presence of TPNH (Fig. 4A, curve 2). The magnitude of this effect of TPNH was examined by calculating the K_I for DPNH in the presence and absence of TPNH from Lineweaver-Burk plots such as shown in Figure 4B. K_I for DPNH was calculated to be 3.9×10^{-5} M. In the presence of 3.3×10^{-5} M TPNH and an equimolar amount of DPNH, the potentiated inhibition resulted in an apparent inhibition constant K_I of 0.76×10^{-5} M. It can be calculated that, under standard assay conditions, 0.1 μmole of DPNH, in the presence of 0.1 μmole of TPNH, exerts the same amount of inhibition as about 0.5 μmole of DPNH alone.

Since impurities might have been present in commercial TPNH samples, TPNH was prepared for inhibition studies in two ways. In the first method, TPNH was generated enzymically in the presence of the DPN-linked isocitrate dehydrogenase reaction mixture by means of glucose 6-phosphate, glucose 6-phosphate dehydrogenase, and TPN^+. Isocitrate oxidation then was initiated by addition of DPN-linked isocitrate dehydrogenase. TPNH was also prepared by reduction of TPN^+ in the TPN-linked isocitrate dehydrogenase system and purified on DEAE-cellulose columns (Pastore and Friedkin, 1961) as previously described (Chen and Plaut, 1963). The purified TPNH had a ratio of optical densities at 260 mμ and 340 mμ of 2.4 at *p*H 7.2. TPNH prepared by either method gave the same results.

The possibility that the effect of TPNH could have been an artifact due to contaminating enzymes was considered. Thus, TPN-linked isocitrate dehydrogenase or glutamate dehydrogenase could decrease the rate of increase in optical density at 340 mμ with time by reoxidizing TPNH with α-ketoglutarate formed in the DPN-linked isocitrate dehydrogenase reaction. Neither enzyme, however, could be demonstrated in the DPN-specific isocitrate dehydrogenase preparation used. Moreover, TPNH potentiated the inhibition by DPNH of the *initial* reaction rate when both nucleotides were present originally (Fig. 4B). It was also conceivable that pyridine nucleotide transhydrogenase could lead to an apparent augmentation in DPNH inhibition by producing more DPNH from DPN^+ and TPNH. However, transhydrogenase did not seem to be present, since DPN^+, TPNH, and enzyme, in the presence of the glucose 6-phosphate dehydrogenase system, did not result in the formation of TPN^+. Also, the amount of inhibition obtained in the presence

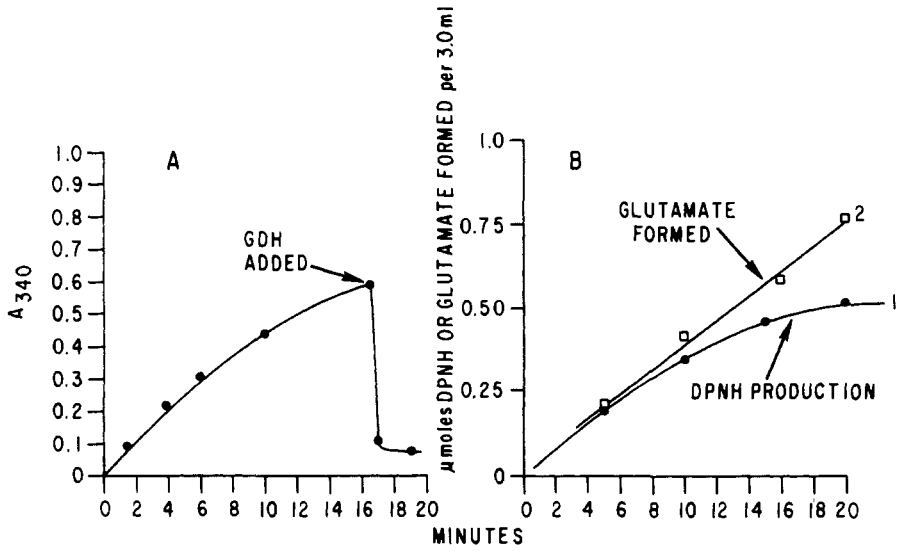

FIG. 5.—Effect of DPNH removal on the rate of isocitrate oxidation. (A) The reaction mixture contained 3.3×10^{-2} M potassium cacodylate buffer, *p*H 6.5; 6.7×10^{-4} M $MnCl_2$; 2.7×10^{-3} M *threo*-D_sL_s-isocitrate; 10^{-2} M NH_4Cl; DPN-linked isocitrate dehydrogenase; and water in a volume of 3.0 ml. At the time indicated, 0.2 mg of crystalline glutamate dehydrogenase (GDH) was added. (B) Curve 1 shows the formation of DPNH in the presence of DPN-specific isocitrate dehydrogenase. The composition of the medium was similar to that in part A. Curve 2, the conditions were similar to those prevailing for curve 1 except that 0.2 mg of glutamate dehydrogenase was present before initiation of the reaction with DPN specific isocitrate dehydrogenase.

of equimolar amounts of TPNH and DPNH was consistently greater than that obtainable by doubling the DPNH concentration in the absence of TPNH.

Like DPNH, ATP and ADPR have been found to inhibit the enzyme competitively with DPN^+. The competitive inhibition constants at *p*H 7.2 of ATP and ADPR were calculated to be 1.5×10^{-4} M and 6.1×10^{-5} M, respectively. Although structurally similar to these nucleotides, ADP did not counteract the inhibition by these compounds.

A number of other nucleotides were found to be inhibitory (Table III). However, when compared to ADPR and ATP, rather large amounts of nucleotides were required for inhibition, thus raising the possibility that the inhibition was due, in part, to chelation of Mn^{++}. This may explain the inhibition of DPN-linked isocitrate dehydrogenase by 2′-AMP, a nucleotide which has been postulated to inhibit TPN-linked enzymes specifically and to have little effect on DPN-linked enzymes (Neufeld *et al.*, 1955).

No effect of ATP, DPN^+, or DPNH on TPN-linked isocitrate dehydrogenase could be shown.

TABLE III
INHIBITION BY VARIOUS NUCLEOTIDES[a]

Nucleotide	Concentration for Approximately 50% Inhibition (M)
IDP	3.0×10^{-3}
GDP	3.0×10^{-3}
ATP	3.5×10^{-4}
ITP	1.7×10^{-3}
UTP	1.7×10^{-3}
2′-AMP	1.5×10^{-2}
3′-AMP	1.5×10^{-2}
5′-AMP	1.5×10^{-2}
ADPR	1.4×10^{-4}

[a] The conditions of incubation were as described under "Assay of Enzymic Activity" except that ADP was omitted. The reactions were initiated with enzyme (specific activity, 400).

Reactivity with DPN Analogs.—Table IV shows the reactivity of DPN-linked isocitrate dehydrogenase system with a number of DPN^+ analogs. The 3-acetylpyridine analog was the only one which oxidized substrate at a significant rate (50% that of DPN^+). The enzyme does not seem to interact with the deamino analog of DPN^+ since there was no reduction of this compound, and it did not inhibit the rate of the isocitrate dehydrogenase reaction with DPN^+. An intact adenosine 5′-pyrophosphoryl moiety seems to be re-

TABLE IV
EFFECT OF VARIOUS PYRIDINE NUCLEOTIDES[a]

Oxidized Nucleotides	Relative Activity[b] (%)
DPN^+	100
α-DPN^+	0
TPN^+	0
Acetylpyridine-DPN^+	50
Thionicotinamide-DPN^+	7
Deamino-DPN^+	0
Pyridinealdehyde-DPN^+	0
NMN^+	0

Reduced Nucleotides	Relative Inhibition[b] (%)
DPNH	100
Acetylpyridine-DPNH	70
Thionicotinamide-DPNH	<10
Deamino-DPNH	0
TPNH	0[c]

[a] All tests done at 25°. For evaluation of the oxidized nucleotides, each cuvet contained in a volume of 3.0 ml: 100 μmoles of Tris-acetate buffer, *p*H 7.2; 4.0 μmoles of $MnCl_2$; 16.0 μmoles of *threo*-D_sL_s-isocitrate; water; and 1.0 μmole of the nucleotide tested. The reaction was started by adding 25 μg of enzyme (specific activity, 400). The reduced nucleotides were tested under similar conditions in the presence of 1.0 μmole of DPN^+. [b] The activity obtained with DPN^+ or DPNH is taken as 100%. [c] See the text.

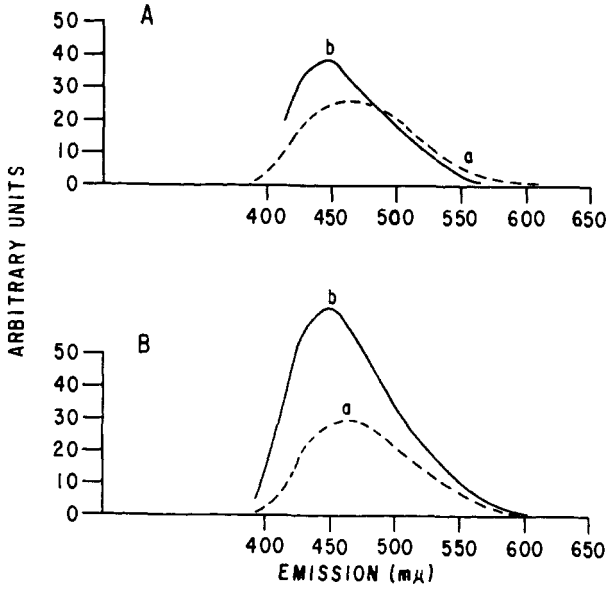

FIG. 6.—Comparison of the fluorescence emission spectra of DPNH and the DPNH-enzyme complex. All solutions contained 0.10 M potassium phosphate, *p*H 7.2. The excitation wavelength was 360 mμ (uncorrected). The dotted curves (a) are difference spectra obtained by subtracting the fluorescence of the buffer alone from that obtained for 3.3×10^{-6} M DPNH, while the solid lines (b) are the spectra for solutions of protein plus 3.3×10^{-6} M DPNH, corrected for the fluorescence of the protein solution alone. The protein was dialyzed for 2 hours at 2° against buffer and any precipitate formed was removed by centrifugation. The protein solutions then were allowed to come to 20°. Each cuvet held 3.0 ml of enzyme solution. 0.1 ml of 1.0×10^{-4} M DPNH was then added and the fluorescence spectra immediately recorded. The addition of DPNH resulted in a 3% dilution of the enzyme solution, but the difference spectra are not corrected for this small error. Part A, 5.4 mg of protein per ml (specific activity, 189). Part B, 0.57 mg of protein per ml (specific activity, 2490).

quired for binding of the nucleotides to the enzyme since ATP, ADPR, and DPNH inhibit competitively with DPN^+ but deamino-DPNH does not.

Fluorimetric Studies on the DPNH-Enzyme Complex.—The inhibition by DPNH suggested that the nucleotide might be bound to the enzyme. Binding of reduced pyridine nucleotides to proteins has been shown in a number of instances to result in an enhancement of the nucleotide fluorescence and a shift of the peak to a lower wavelength (see Udenfriend, 1962). Such an effect has been obtained by Langan (1960) for TPNH and the TPN-linked isocitrate dehydrogenase. Similar evidence for binding has been obtained for the DPN-linked enzyme (Fig. 6). Enzyme preparations of different purity were used in the experiments depicted in Figure 6, but the emission peak of DPNH in both instances was shifted from 460 mμ to 444 mμ. The nucleotide fluorescence intensity increased 46% in the presence of 1020 units of the less pure preparation (specific activity, 189) (Fig. 6A), whereas 1420 units of the highly purified enzyme (specific activity, 2490) enhanced the intensity 120% (Fig. 6B) although there was much less protein present. The lack of correlation of enzymic activity and fluorescence enhancement suggests that some quenching may have occurred with the less pure enzyme solution.

DISCUSSION

The present purification procedure yields DPN-linked isocitrate dehydrogenase with a specific activity of 4500. Previously, a preparation with a specific activity of 1410 was reported (Plaut and Sung, 1954). However, the actual increase in purity of the product is only about 2-fold since the present study employed conditions of assay which gave 67% more activity than the previous procedure. Testing of the material with a specific activity of 4500 (Table I) by ultracentrifugation indicates that the enzyme is approaching a high state of purity since the activity seems to reside in a major component comprising about 85% of the protein.

The DPN-linked isocitrate dehydrogenase appears to be a larger molecule than the TPN-specific enzyme, which was found to have a molecular weight of about 60,000 (Moyle and Dixon, 1956; Siebert *et al.*, 1957). Although it was impossible to do diffusion studies on the DPN-linked enzyme with the small amount of protein available, the molecular weight may be in the range of $3–4 \times 10^5$. A specific activity as high as 5520 units/mg was observed in the course of hydroxylapatite chromatography of certain preparations of enzyme. If this were the specific activity of the pure enzyme, and 3×10^5 were the molecular weight, the turnover number of the enzyme would be 8.0×10^3 moles of DPNH formed per minute per mole of enzyme or 2.7×10^3 moles/minute/10^5 g protein. This can be compared to a turnover number for the TPN-specific isocitrate dehydrogenase of 3.5×10^3 moles TPNH formed per minute per mole of enzyme (Siebert *et al.*, 1957).

The activation of this enzyme by ADP is comparable to nucleotide stimulation of several other enzymes. Such effects are apparently catalytic in nature, since the nucleotide does not appear to be consumed in the enzymic reaction. 5′-AMP has long been known to be essential for the activity of phosphorylase b (see Cori, 1940; Brown and Cori, 1961; Krebs and Fischer, 1962). 2′-AMP has been found to stimulate bacterial pyridine nucleotide transhydrogenase (Kaplan *et al.*, 1953). It was noted by Rall *et al.* (1957) that the formation of active liver phosphorylase was mediated by a heat-stable, dialyzable factor. This factor was later identified as 3′,5′-cyclic-AMP (Sutherland and Rall, 1958). The activation of dephosphophosphorylase kinase of liver has in fact become the basis of a sensitive assay for 3′,5′-cyclic-AMP (Rall and Sutherland, 1958). In the case of the corresponding muscle enzyme, phosphorylase b kinase, Krebs *et al.* (1959) found that activation by ATP and Mg^{++} was stimulated by 3′,5′-cyclic-AMP. Stimulation by 3′,5′-cyclic-AMP has also been noted for the phosphofructokinase of *Fasciola hepatica* (Mansour and Mansour, 1962). Cormier (1962) has identified 3′,5′-diphosphoadenosine as the cofactor in the luminescence reaction of the sea pansy, *Renilla reniformis;* but in this instance, the nucleotide may be reacting with luciferin to give a heat-labile intermediate. Bovine liver glutamate dehydrogenase, which is affected by a number of nucleotides, is stimulated markedly by ADP, and to a lesser extent by adenosine and 5′-AMP, when reduced pyridine nucleotide is used as coenzyme (Frieden, 1959); ADP was shown to favor aggregation of subunits of the enzyme. ADP likewise protects the enzyme from inhibition and disaggregation by steroids (Yielding and Tomkins, 1962). On the other hand, GTP is inhibitory (Frieden, 1962). It has been postulated that nucleotide stimulation of glutamate dehydrogenase involves configurational changes which also favor aggregation of catalytically active subunits (Fisher *et al.*, 1962; Frieden, 1963). Muntz (1953) discovered that dog brain 5′-adenylate deaminase activity was stimulated by ATP. The purified deaminase was found

to be completely dependent on catalytic amounts of ATP, which did not partake in any exchange reaction with 5′-AMP (Mendicino and Muntz, 1958). Bovine brain 5′-adenylate deaminase was also found to be stimulated by ATP; but, in contrast to the dog brain enzyme, considerable activity was present in the absence of ATP (Weil-Malherbe and Green, 1955). ATP has been shown to be a required cofactor for the hydrolase which splits 5′-AMP to adenine and ribose 5-phosphate in *Azotobacter vinelandii* (Hurwitz *et al.*, 1957). Isotope-labeling experiments showed no interconversion between ATP and the compounds involved in the enzymic reaction. ATP was replaceable by adenosine tetraphosphate, pyrophosphate, and tripyrophosphate, but not by a number of other nucleoside triphosphates. Gerhart and Pardee (1962) have recently reported stimulation of the purified aspartate transcarbamylase of *Escherichia coli* by ATP or d-ATP; the stimulation by these nucleotides under their assay conditions amounted to 80% and 62%, respectively.

The ADP stimulation of heart DPN-linked isocitrate dehydrogenase seems to differ from the 5′-AMP stimulation of the yeast enzyme (Kornberg and Pricer, 1951) in two important aspects; i.e., ADP does not seem to be firmly bound to the heart enzyme, and considerable activity can be demonstrated even without ADP, whereas the yeast enzyme showed almost absolute dependence on 5′-AMP. However, it should be noted that at low isocitrate concentrations, and above *p*H 7.0, the heart enzyme has little activity unless ADP is present (Fig. 2A and Fig. 3). This is due to the decrease in the apparent K_m of isocitrate in the presence of ADP. Kornberg and Pricer, in their study of the yeast enzyme, employed only low amounts of *threo*-D,-isocitrate (0.83×10^{-4} M) and did not report the effect of 5′-AMP on their enzyme at higher isocitrate levels. However, as noted above, the rate of reaction of the heart enzyme at 1×10^{-4} M isocitrate is only 1–2% of the ADP-stimulated reaction. Moreover, a recent preliminary communication (Atkinson and Hathaway, 1963) indicates that at higher isocitrate concentrations the yeast DPN-linked isocitrate dehydrogenase is *not* dependent on 5′-AMP. Thus the nucleotide stimulation of the yeast enzyme may be qualitatively quite similar to that of the beef heart enzyme described in the present work.

A similar alteration of the K_m for substrate by ATP was found by Gerhart and Pardee (1962) in the case of aspartate transcarbamylase. Other instances are known in which K_m is lowered by an activator of an enzyme. For example, Glaser and Brown (1957) found that the particulate chitin synthetase of *Neurospora crassa* was stimulated by *N*-acetylglucosamine resulting in a decrease in K_m for UDP-*N*-acetylglucosamine. K^+ activation of carbamylphosphate synthetase also resulted in lowered Michaelis constants for NH_4^+, ATP, and acetylglutamate (Marshall *et al.*, 1961). Glucose 6-phosphate stimulation of UDP-glucose-glycogen transglucosylase also reduced K_m for UDP-glucose (Rosell-Perez *et al.*, 1962; Kornfeld and Brown, 1962).

The activation of DPN-linked isocitrate dehydrogenase by ADP could well be of significance, since there is some evidence that isocitrate concentration within mitochondria may be in the region where the ADP effect would be most prominent. Bellamy (1962) has estimated the intramitochondrial content of citrate in liver mitochondria to be about 5 μmoles per g. The isocitrate concentration would then be about 3×10^{-4} M if one assumes that the aconitase-catalyzed equilibrium between the tricarboxylic acids obtains (Krebs, 1953). At *p*H 7.2 in the presence of such a low concentration of isocitrate, bovine heart DPN-specific isocitrate dehydrogenase shows virtually absolute dependency on ADP. It is not known, however, whether the tricarboxylic acid contents of heart and liver mitochondria are comparable.

The fact that DPN-linked isocitrate dehydrogenase is, on the one hand, activated by ADP, and, on the other hand, inhibited by ATP and DPNH, suggests a possible regulatory role of the nucleotides in mitochondrial oxidation of certain substrates. Under conditions favoring respiration in mitochondria, it has been shown that the ratios [DPNH]-[DPN$^+$] and [ATP]-[ADP] are low (see Chance and Williams, 1956). These conditions typify Chance's "state 3," and under these circumstances the oxidation of isocitrate by DPN-linked isocitrate dehydrogenase would be favored since ADP is stimulatory and DPN$^+$ competitively counteracts the inhibitory effect of DPNH. Conversely, under anaerobic conditions, or where phosphate acceptor concentrations are low, the [DPNH]-[DPN$^+$] and [ATP]-[ADP] ratios are high ("state 4" of Chance). Under these conditions, DPN-linked isocitrate dehydrogenase activity would be inhibited because the lack of ADP would diminish the affinity of the enzyme for isocitrate, and the inhibition would be potentiated further by the action of high concentrations of DPNH and of ATP at the site of DPN$^+$. The inhibition by DPNH may be a highly effective mechanism for regulation of DPN-specific isocitrate dehydrogenase under physiological conditions, because the apparent affinity of the enzyme for DPNH is markedly increased by TPNH (Fig. 4). The extent of inhibition of the enzyme by pyridine nucleotides would then be a function of the levels of DPN$^+$, DPNH, and TPNH. Alterations in the [ATP]-[ADP] and [DPNH]-[DPN$^+$] ratios in mitochondria could thus serve as a mechanism regulating the rate of substrate oxidation at the level of isocitrate. Some evidence exists for such a mechanism. A study of the oxidative metabolism of cardiac mitochondria showed that citrate oxidation was enhanced by lowering the ATP concentration and by adding DPN$^+$ to the incubation medium (Plaut and Plaut, 1952).

In connection with the possible regulatory role of nucleotides in cellular isocitrate oxidation it is of interest that the activity of TPN-linked isocitrate dehydrogenase from bovine heart is not influenced by ADP, ATP, DPN$^+$, or DPNH.

At the present time one can only speculate as to the mechanism whereby ADP stimulates DPN-linked isocitrate dehydrogenase of heart. While the nucleotide obviously influences the K_m for isocitrate, it is possible that a conformational change of the protein may be induced also. Such a change may be evidenced by an altered state of aggregation of the enzyme molecule, as in the case of ADP-treated glutamate dehydrogenase (Frieden, 1959) or citrate-stimulated adipose tissue acetyl CoA carboxylase (Vagelos *et al.*, 1963). Further studies on this possibility are in progress.

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