ENZYME SYSTEMS IN THE MYCOBACTERIA

I. The Isocitric Dehydrogenase

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Millman and Youmans (1954, 1955) have recently reported that a cell-free extract of *Mycobacterium tuberculosis* strain H37Ra, when supplemented with coenzymes and methylene blue, takes up oxygen slowly in the presence of various intermediates of the tricarboxylic acid cycle. There was no correlation made between the amount of substrate disappearing and of oxygen consumed; oxidation products were not identified. Although this and other (Geronimus, 1949; Ochoa, *et al.*, 1951) presumptive evidence exists for the presence of the tricarboxylic acid cycle in strains of *M. tuberculosis*, there has been no direct demonstration of this metabolic sequence in either the human pathogens or their avirulent variants.

The present report is concerned with the description of a TPN-specific[1] isocitric dehydrogenase isolated from cell-free extracts of *Mycobacterium tuberculosis* var. *hominis* strain H37Ra.

The TPN-isocitric dehydrogenase catalyzing the over-all reaction

(1) D-isocitrate + $TPN^+ \rightleftharpoons$ α-ketoglutarate + TPNH + H^+ + CO_2

has been shown to be widely distributed in animal, plant and bacterial cells (Wagner-Jauregg and Rauen, 1935; Adler *et al.*, 1939; von Euler *et al.*, 1939; Ochoa, 1945; Lynen and Scherer, 1948; Ceithaml and Vennesland, 1949). Ochoa (1945) has purified this enzyme and established its stoichiometry. Ochoa (1948) further showed that, as had been postulated earlier by Martius (1937), the conversion of isocitrate to α-ketoglutarate proceeds through the intermediate formation of oxalosuccinate. The reversibility of reaction (1) has been established by Ochoa (1948) and by Kornberg and Pricer (1951), who used, respectively, a heart muscle preparation and a yeast preparation. Moyle and Dixon (1955) recently purified the TPN-isocitric dehydrogenase of heart muscle to the stage of electrophoretic homogeneity and demonstrated the identity of the dehydrogenase and the decarboxylase, a conclusion reached earlier by Grafflin and Ochoa (1950) on the basis of studies of a less purified enzyme preparation.

[1] The following abbreviations are used: triphosphopyridine nucleotide, oxidized and reduced, TPN and TPNH; diphosphopyridine nucleotide, DPN; Tris(hydroxymethyl)aminomethane, Tris.

METHODS AND MATERIALS

Materials. TPN (purity 0.95 or higher) was obtained from the Pabst Laboratories and Sigma Chemical Co.; α-ketoglutaric acid (mp 115C) and DL-isocitric acid from Nutritional Biochemicals Corp.; bovine serum albumin Fraction V from Pentex, Inc. A stock culture of *M. tuberculosis* strain H37Ra was kindly supplied by Dr. G. P. Youmans of the Department of Bacteriology, Northwestern University School of Medicine.

Bacterial preparations. *M. tuberculosis* was grown as a surface culture on a modified Proskauer and Beck liquid medium to which had been added sufficient 5 per cent bovine serum albumin solution containing 0.95 per cent oleic acid to make a final albumin concentration of 0.15 per cent (Youmans and Karlson, 1947). The medium was inoculated with approximately 1 cm^2 of a three-week-old surface culture. After four weeks of growth at 38 C the entire surface of the medium (220 cm^2) is covered with the cell mass. The cells are collected on a coarse porosity sintered glass filter by filtration, washed thoroughly with distilled water and suspended in 0.01 M phosphate buffer of pH 7.0. In general, the contents of 15 culture bottles are harvested at one time.

Enzyme purification. All procedures were carried out at 1 to 3 C. The washed cell mass,

TABLE 1

Purification of the TPN-isocitric dehydrogenase of H37Ra

Fraction	Volume (ml)	Protein			Units		
		Mg per ml	Total mg	Per cent recovery	Specific activity	Total units	Per cent recovery
Crude extract.....	510	15.4	7850	100	0.18	1400	100
AS-1.....	176	25.9	4590	58	0.18	820	58
AS-2.....	110	35.8	3940	50	0.19	765	55
AS-3.....	9.5	59.0	560	7	0.72	405	29

suspended in buffer, is exposed to 10 kc sonic oscillation for 35 to 40 minutes. During this period the temperature inside the treatment cup is held at 1 to 4 C by circulation of −6 C coolant through the outer jacket of the container. The suspension is centrifuged at 15,000 × G for 120 minutes and the residue, consisting of unbroken cells and cellular debris, is discarded. The opalescent supernatant solution is sterilized by filtration through an ultra-fine porosity sintered glass filter. The crude cell-free extract may be stored at −15 C for several months without appreciable loss of isocitric dehydrogenase activity.

To each 100 ml of cell-free extract is added 20 g of ammonium sulfate. After centrifugation at 4,000 × G for 30 minutes the residue is discarded and the clear supernatant solution is saturated with ammonium sulfate by the addition of about 53 g of solid reagent per original 100 ml of extract. The suspension is filtered, the filtrate discarded and the residue, after removal from the paper, is dissolved in 0.02 M $KHCO_3$ (AS-1). To AS-1 is added sufficient saturated ammonium sulfate solution (pH 7) to reach 0.30 saturation. The small precipitate which forms is removed by centrifugation and discarded. More saturated ammonium sulfate solution is added to the supernatant solution until 0.65 saturation is reached. The precipitate (AS-2) is removed by centrifugation, dissolved in and dialyzed against 0.02 M $KHCO_3$. AS-2 is further fractionated with ammonium sulfate.[2] The fraction precipitating between 0.40 and 0.50 saturation is retained (AS-3). Solutions of the enzyme at this level of purification show a slow decline in activity on storage at −15 C. Attempts at further purification of the enzyme (gels, alcohol, heat denaturation) have not been successful. A typical enzyme fractionation is shown in table 1.

The highest specific activity obtained by the procedure described above was 1.0; specific activities of 0.7 to 0.8 are routinely obtained. This activity is equal to that of the best preparations of the TPN-isocitric dehydrogenase of baker's yeast, as prepared by Kornberg and Pricer (1951).

Enzyme assay. The mixture for assay of the TPN-isocitric dehydrogenase contains Tris buffer of pH 7.3 (10 μmoles), TPN (1.0 μmole), $MnCl_2$ (5 μmoles), isocitrate (3 μmoles) and enzyme (10 to 50 μg). The final volume is made to 1.0 ml with water. Assay temperature is 21 C. The reaction is started by the addition of the isocitrate. The increase in optical density at 340 mμ resulting from TPN reduction is followed in the Beckman DU spectrophotometer. Three readings are taken at one-minute intervals. Linearity of TPN reduction is generally observed when the change in optical density at 340 mμ is in the range of 0.01 to 0.15 per minute. A unit of enzyme activity is defined as that amount causing the reduction of 1.0 μmole of TPN per minute. Specific activity is defined as units of enzymatic activity per mg protein. The molecular extinction coefficient of TPN is taken as 6.22×10^6 cm^2 × mole^{-1} (Horecker and Kornberg, 1948).

Protein concentration was measured by the biuret reaction (Gornall, *et al.*, 1949). α-Ketoglutarate was determined colorimetrically as the 2,4-dinitrophenylhydrazone (Friedemann and Haugen, 1943).

RESULTS

Characteristics of the assay system. The dependence of the velocity of oxidation of iso-

[2] Results have consistently demonstrated that if the intermediate fractionation step resulting in AS-2 is omitted, then the third purification step results in a much poorer preparation.

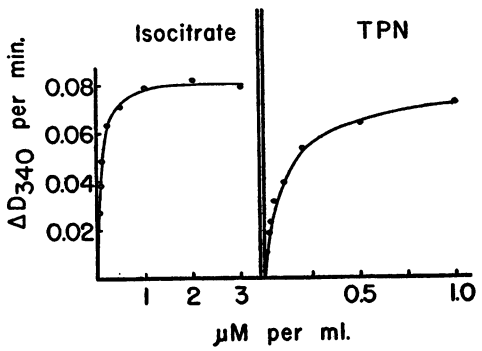

Figure 1. Activity of the TPN-isocitric dehydrogenase assay system as a function of isocitrate and TPN concentration.

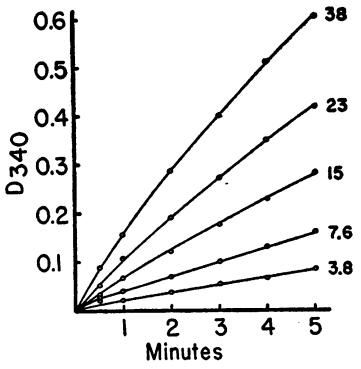

Figure 2. Activity of the TPN-isocitric dehydrogenase assay system as a function of time and concentration of enzyme. Figures represent μg of protein. Ordinate represents total change in optical density at 340 mμ corrected for a blank containing no enzyme.

citrate on the concentration of isocitrate and TPN, respectively, is shown in figure 1. The linearity of the system with respect to time and enzyme concentration is shown in figure 2. Figure 3 shows the requirement of the system for Mn^{++} and the partial substitution by Mg^{++} for this requirement. The pH curve for the TPN-isocitric dehydrogenase is shown in figure 4.

Formation of α-ketoglutarate. The correspondence between α-ketoglutarate and TPNH formation is shown in table 2. The absorption spectrum of the dinitrophenylhydrazone of the TPN-isocitric dehydrogenase reaction product is identical with that of the dinitrophenylhydrazone of known authentic α-ketoglutaric acid.

Reversibility of isocitric oxidation. Figure 5 shows an experiment in which isocitric acid is oxidized with TPN as electron acceptor. After this reaction had gone to completion first CO_2 (as $KHCO_3$ (20 μmoles) saturated with CO_2) and then α-ketoglutarate (15 μmoles) were added. Reversal of reaction (1) is shown by the decrease in optical density at 340 mμ. The same results obtain if the CO_2 and α-ketoglutarate are added

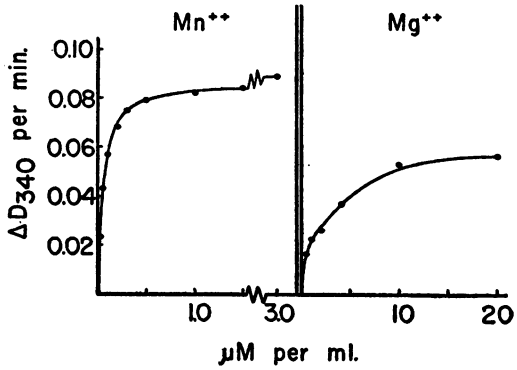

Figure 3. Activity of the TPN-isocitric dehydrogenase assay system as a function of metal ion concentration.

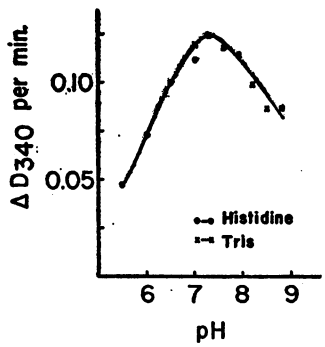

Figure 4. Activity of the TPN-isocitric dehydrogenase assay system as a function of pH.

TABLE 2

Formation of α-ketoglutarate from isocitrate

μmoles TPN at start	0.20	0.40
Δ μmoles TPNH (1)	0.14	0.24
Δ μmoles α-ketoglutarate (2)	0.13	0.27
(1)/(2)	1.08	0.89

Each tube contained the following: Tris buffer of pH 7.3 (30 μmoles), TPN as shown, $MnCl_2$ (15 μmoles), isocitrate (10 μmoles), enzyme of specific activity 0.5 (65 μg) and H_2O to 3.0 ml. The tubes were incubated at 38 C for 10 minutes, the optical density at 340 mμ read, and their contents assayed for α-ketoglutaric acid. The reactions were run in duplicate and averaged for this table.

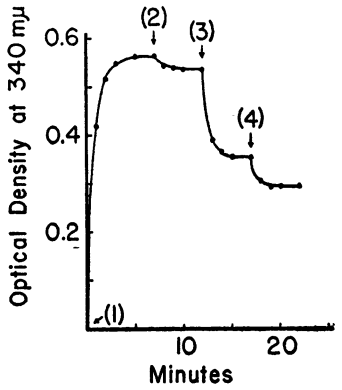

Figure 5. Demonstration of the reversibility of reaction (1). The microcuvette contained Tris buffer of pH 7.3 (20 μmoles), $MnCl_2$ (1 μmole), isocitrate (0.6 μmole), enzyme of specific activity 0.4 (0.56 mg) and H_2O to 0.95 ml. The reaction was started by the addition of 0.1 μmole TPN (point 1). Further additions were 20 μmoles $KHCO_3$ (2), 15 μmoles α-ketoglutarate (3) and 30 μmoles $KHCO_3$ (4). Reaction carried out at 21 C. Reaction shown has been corrected by a no-substrate blank.

in small increments instead of these large amounts; the decrease in optical density is, however, correspondingly smaller.

K_s determinations. K_s values (Michaelis and Menten, 1913) for isocitrate, TPN and Mn^{++} were found to be 5×10^{-5}, 5.5×10^{-5} and 5.5×10^{-5} moles × $liter^{-1}$, respectively.

Action of inhibitors. The TPN-isocitric dehydrogenase is not inhibited by 1×10^{-3} M fluoride or by 1×10^{-2} M arsenite. Preincubation for 15 min of the assay mixture without substrate but with inhibitor did not change these results. Arsenite slightly stimulated the rate of reaction. *p*-Chloromercuribenzoate at a concentration of 2×10^{-5} M totally inhibits the enzyme and at a concentration of 2×10^{-6} M inhibits the enzyme by about 45 per cent.

Coenzyme specificity. DPN cannot substitute for TPN in this system. No detectable reaction obtains when 20 μmoles of DPN are substituted for 1.0 μmole of TPN in the assay system.

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SUMMARY

The triphosphopyridine nucleotide-isocitric dehydrogenase of *Mycobacterium tuberculosis* strain H37Ra has been isolated and investigated. This enzyme is similar to that previously isolated from animal tissues with respect to metal ion activation, pH optimum, K_s values and coenzyme specificity. α-Ketoglutarate is formed quantitatively from isocitrate. The enzyme is inhibited by *p*-chloromercuribenzoate.

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