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ENZYMES OF THE PYRIMIDINE PATHWAY IN *ESCHERICHIA COLI*

II. INTRACELLULAR LOCALIZATION AND PROPERTIES OF DIHYDROOROTIC DEHYDROGENASE

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ABSTRACT

TAYLOR, W. HERMAN (Portland State College, Portland, Ore.), AND MARY L. TAYLOR. Enzymes of the pyrimidine pathway in *Escherichia coli*. II. Intracellular localization and properties of dihydroorotic dehydrogenase. J. Bacteriol. **88**:105-111. 1964.—Intracellular localization of three enzymes of the pyrimidine pathway in *Escherichia coli* was studied. Dihydroorotic dehydrogenase was found to be associated with the membrane portion of lysed spheroplasts. Centrifugal fractionation of cell-free extracts showed all the dihydroorotic dehydrogenase activity to be associated with large structures, probably cell wall-membrane fragments. In contrast, all orotidylic decarboxylase activity was found in the cytoplasm in both lysed spheroplasts and cell-free extracts. Aspartate transcarbamylase activity appeared to be particulate in repressed cells, but only 25% was particulate in derepressed cells. Dihydroorotic dehydrogenase was shown to be bound to oxidative particles by oxygen uptake and orotate production from dihydroorotate. A ferricyanide reduction assay, suitable for measuring soluble and particulate enzyme, was devised for dihydroorotic dehydrogenase. Soluble dihydroorotic dehydrogenase was prepared by use of deoxycholate. A 20-fold purification of the enzyme compared to whole-cell activity was achieved by ammonium sulfate fractionation of the deoxycholate-soluble enzyme. Although cytochromes were implicated by cyanide inhibition of aerobic orotate production by particles, the purified enzyme appeared to be separated from the cytochromes, as shown by lack of cyanide inhibition in the ferricyanide assay. The purified soluble enzyme did not react in the aerobic assay previously used by others for assay of this enzyme. In contrast to the degradative dihydroorotic dehydrogenases reported by other workers, the biosynthetic dihydroorotic dehydrogenase of *E. coli* did not link to pyridine nucleotides.

The finding that lysozyme spheroplasts of *Escherichia coli* would not form dihydroorotic dehydrogenase upon release from uracil repression (Taylor and Novelli, 1964) prompted us to investigate the nature of this enzyme. Previous work with the biosynthetic dihydroorotic dehydrogenase from *E. coli* employed an aerobic assay, in which the rate of orotate production from dihydroorotate (DHO) was measured in crude extracts or toluene-treated cells (Yates and Pardee, 1957). This aerobic assay provided no external electron acceptor except oxygen, and no mention was made of oxygen dependence.

This paper reports a new assay for dihydroorotic dehydrogenase and contains our findings on the cellular localization, properties, and purification of dihydroorotic dehydrogenase from *E. coli*. Also, data on the intracellular distribution of two other enzymes in the pyrimidine pathway are reported. A preliminary report on a portion of this work has appeared (Taylor and Novelli, 1961).

MATERIALS AND METHODS

Mutants and growth conditions. *E. coli* K-12, 496; *E. coli* 15, R185-482; and the culture media were previously described (Taylor and Novelli, 1964). *E. coli* K-12, 496 cells were released from repression by incubation in a medium containing no uracil. *E. coli* 15, R185-482 cells were released from repression by incubation in medium containing 0.001 M sodium orotate and no uracil.

Bacterial extracts. Extracts were prepared from derepressed cells with a French press (American Instrument Co., Inc., Silver Spring, Md.) with the use of 5,000 psi pressure or in a Raytheon 9-kc sonic oscillator.

Enzyme assays and chemical determinations. Orotidylic acid decarboxylase and aspartate

transcarbamylase were assayed as previously described (Taylor and Novelli, 1964).

Dihydroorotic dehydrogenase was assayed by the aerobic method of Yates and Pardee (1957) or by the ferricyanide reduction method described as follows. Assay mixtures contained 300 μmoles of potassium phosphate, pH 8.0 (when diluted tenfold); potassium ferricyanide, 3 μmoles; potassium cyanide, 30 μmoles; sodium dihydroorotate, 4 μmoles; and enzyme and water to make a total volume of 3 ml. The rate of optical density decrease due to ferricyanide reduction was measured at room temperature in a Klett-Summerson colorimeter with filter 42. The reduction of 1 μmole of ferricyanide caused a decrease of 73 Klett units; 1 μmole of orotate produced was equivalent to reduction of 2 μmoles of ferricyanide. The ferricyanide assay was used except where indicated. The optimal pH for the aerobic assay was approximately 7.5, whereas the pH optimum for the ferricyanide assay was 8.0.

One unit of enzyme is defined as the amount of enzyme which will produce 1 μmole of product (orotic acid, ureidosuccinic acid, or uridylic acid) per hr under the stated conditions. Specific activity is listed as units per milligram of protein. Protein was measured by the Folin reagent (Lowry et al., 1951), with a bovine serum albumin standard.

Spheroplast formation. Spheroplasts were prepared by the lysozyme and ethylenediaminetetraacetate method of Taylor and Novelli (1964). Efficiency of spheroplast formation was determined by phase microscopy and by lysis of spheroplasts in water.

Treatment of samples for enzyme assay. Cell suspensions were treated with toluene (0.05 ml per ml of cell suspension) for 5 min at room temperature, sedimented by centrifugation, and resuspended in water. Spheroplasts were sedimented by centrifugation and the pellets were resuspended in water. Lysis occurred immediately. Spheroplast lysates were separated into cytoplasmic and membrane fractions by centrifugation at 30,000 × *g* for 65 min.

Fractionation of dihydroorotic dehydrogenase. Washed particle preparations from two French press extracts (1,050 mg of protein) were suspended to 20 ml in 0.01 M tris(hydroxymethyl)-aminomethane (tris; pH 7.8) containing 0.0015 M 2-mercaptoethanol. The particle suspension was mixed with 10 ml of 10% sodium deoxycholate (DOC) at 0 C. After 10 min at 0 C, the mixture was diluted to 48 ml with water, and was centrifuged at 100,000 × *g* for 2 hr in a Spinco model L ultracentrifuge. To remove DOC, the supernatant (40 ml) was dialyzed for 16.5 hr against three 1-liter changes of 0.01 M potassium phosphate (pH 7.4) containing 0.0015 M 2-mercaptoethanol. The dialyzed supernatant fluid was then fractionated with saturated ammonium sulfate solution adjusted to pH 8.7 with ammonium hydroxide. Before fractionation, the dialyzed supernatant fluid was adjusted to 0.1 M with respect to tris (pH 8.7). Precipitates were collected between 0.1 to 0.2, 0.2 to 0.3, 0.3 to 0.4, and 0.4 to 0.5 ammonium sulfate saturation. At 0.5 saturation, 20% of the dihydroorotic dehydrogenase activity remained soluble. Only 15% of the enzyme activity was lost during the ammonium sulfate fractionation.

RESULTS

Electron acceptors for DHO oxidation. Particles separated from sonic or French press extracts converted DHO to orotate in air, and consumed 0.5 μmoles of O_2 per μmole of DHO added. In a nitrogen atmosphere or in the presence of sodium pyrogallate, particle preparations produced almost no orotate from DHO. Thus, the particulate enzyme seemed to react directly with oxygen. The involvement of cytochromes in oxidation of DHO to orotate was suggested by the 80% inhibition of DHO oxidation by potassium cyanide (Table 1, experiment 1). To determine whether the enzyme itself was a cytochrome, rather than being bound to a cytochrome-containing structure, we tested ferricyanide as an alternate electron acceptor (Table 1, experiment 2). Ferricyanide was reduced by DHO in the presence of oxygen, but cyanide stimulated the flow of electrons to ferricyanide. When ferricyanide was the electron acceptor, cyanide caused a 40% inhibition of orotate production, but 2 μmoles of ferricyanide were reduced for each micromole of orotate formed. Thus, the aerobic assay may involve cytochromes as electron carriers, but the electrons flow exclusively to ferricyanide when cytochrome reduction is blocked. The cytochrome is undoubtedly associated with the particle and not the enzyme itself, because cyanide caused almost no stimulation of ferricyanide reduction by soluble purified enzyme (Table 2).

Particles and soluble enzyme preparations failed to catalyze the oxidation of DHO by either nicotinamide adenine dinucleotide (NAD) or

nicotinamide adenine dinucleotide phosphate (NADP). Also, neither reduced NAD (NADH) nor reduced NADP (NADPH) was oxidized by orotate in the presence of either enzyme preparation with the procedures of Friedmann and Vennesland (1960).

Ferricyanide reduction assay. Because the measurement of ferricyanide reduction was simple and convenient and the stoichiometry of ferricyanide reduction was correct (Table 1), an assay procedure involving ferricyanide reduction was devised for dihydroorotic dehydrogenase. Conditions for the standard assay are outlined in Materials and Methods. Requirements of the assay are shown in Table 2. The addition of potassium cyanide was required for equivalence of ferricyanide reduction and orotate production in crude preparations, and was always included in reaction mixtures to be certain that particulate as well as soluble enzyme was measured. The pH optimum for the assay was approximately 8.0, and the rate was slightly higher in phosphate than in tris buffer. The rate of ferricyanide reduction was linear with enzyme concentration when conducted within a range causing 5 to 15 Klett units change per minute.

Intracellular distribution of enzymes. All of the dihydroorotic dehydrogenase activity in spheroplast lysates could be found in membrane or "ghost" fractions isolated by differential centrifugation. This suggested that the location of the enzyme in cells was in the membrane. Table 3 shows the distribution of the enzyme in centrifugal fractions of a French press extract. The crude extract was treated with deoxyribonuclease and ribonuclease prior to centrifugation. The majority of the enzyme activity was present in the structures sedimented at 10,000 and 30,000 × *g*. All of the enzyme in the supernatant fluid at 30,000 × *g* could be sedimented by further centrifugation at 100,000 × *g*. Repeated washing of the 10,000 and 30,000 × *g* pellets resulted in an increased specific activity for dihydroorotic dehydrogenase, indicating a firm binding between the enzyme and particle.

TABLE 1. *Effect of cyanide on the activity of particulate dihydroorotic dehydrogenase**

Expt	Addition to reaction mixture	Orotate formed†	Ferricyanide reduced†
1	None	4.55	
	KCN	0.55	
2	Ferricyanide	4.76	2.65
	Ferricyanide and KCN	2.86	5.35

* Reaction mixtures contained 200 μmoles of potassium phosphate (pH 7.4); particles were washed and resuspended in 0.01 M tris (pH 7.6), containing 0.005 M magnesium acetate, 0.0015 M 2-mercaptoethanol, and 6 μmoles of DHO in a total volume of 3 ml. Where indicated, 20 μmoles of KCN or 10 μmoles of potassium ferricyanide were added. Rates of orotate formation and ferricyanide reduction were estimated from optical density changes at 280 and 400 mμ, respectively, in a Beckman DU spectrophotometer. In experiment 2, the two rates were measured simultaneously in the same cuvette.

† Results expressed as μmoles per hr per mg of protein.

TABLE 2. *Requirements of the ferricyanide reduction assay**

System	Rate of ferricyanide reduction†
Complete	7.0
Minus KCN	6.3
Minus DHO	0.7
Minus enzyme	0.7
Minus enzyme, plus boiled enzyme	0.5

* Ammonium sulfate purified enzyme, 0.054 mg of protein, at a specific activity of 67.6 (Table 6), was used in the standard assay (Materials and Methods).

† Results expressed as μmoles per hr.

TABLE 3. *Distribution of dihydroorotic dehydrogenase in centrifugal fractions of an extract*

Fraction	Protein	Dihydroorotic dehydrogenase	
		Total units	Specific activity
	mg		
Whole extract	4,280	14,800	3.46
Pellet (2,400 × *g*, 45 min)	470	2,000	4.26
Combined pellets (10,000 × *g*, 60 min, and 30,000 × *g*, 60 min)	2,130	10,500	4.93
Supernatant (30,000 × *g*, 60 min)	2,160	5,340	2.47

TABLE 4. *Distribution of enzymes in centrifugal fractions of an extract of mutant R185-482*

Fraction	Enzyme activity					
	Dihydroorotic dehydrogenase		Orotidylic decarboxylase		Aspartate transcarbamylase	
	Total units	Specific activity	Total units	Specific activity	Total units	Specific activity
Crude extract	23.2	7.5	5.9	1.97	75.5	24.4
Pellet (30,000 × *g*, 65 min)	16.2	11.3	0.0*	0.0	19.9	15.4
Supernatant (30,000 × *g*, 65 min)	7.2	4.3	6.0	3.6	57.0	34.2

* As few as 0.02 total units could have been detected.

TABLE 5. *Effect of deoxycholate on the activity of dihydroorotic dehydrogenase**

Treatment	Enzyme activity†
Water	
Uncentrifuged	130
Supernatant	5
Pellet	152
Deoxycholate	
Uncentrifuged	47
Supernatant	50
Pellet	4

* A washed particle preparation (see text) was mixed with an equal volume of 10% deoxycholate (DOC), or water, and kept at 0 C for 10 min; the mixture was diluted sixfold with water and centrifuged at 100,000 × *g* for 2 hr. Activities listed are for the ferricyanide assay. DOC destroyed all activity as measured by the aerobic assay.

† Expressed as the number of units per fraction.

The intracellular distribution of two other enzymes in the pyrimidine pathway was also studied. Cells of mutant R185-482 were released from repression in the presence of orotate and broken with the French Press. This extract was centrifuged and the fractions were analyzed for enzyme activity (Table 4). In mutant R185-482 (as in mutant 496), dihydroorotic dehydrogenase was particulate. Orotidylic decarboxylase was completely soluble, and 75% of the aspartate transcarbamylase activity was found in the supernatant fluid. In toluene-treated cells, the specific activity of dihydroorotic dehydrogenase was 13.4, and the specific activity of aspartate transcarbamylase was 43.8. Comparison of these values with the data for whole extracts (Table 4) indicates some enzyme destruction during preparation of extracts. Orotidylic decarboxylase was not measured in toluene-treated cells. Although not evident in Table 4, aspartate transcarbamylase was almost all particulate in repressed cells. Thus, aspartate transcarbamylase enzyme may be released into the cytoplasm during derepression.

Preparation and purification of soluble dihydroorotic dehydrogenase. Particulate enzyme, prepared by centrifugation and washing of pellets, was neither made soluble nor inactivated by treatment with the following agents: ethylenediaminetetraacetate, sonic oscillation, lipase, deoxyribonuclease, or ribonuclease. Treatment with trypsin or DOC resulted in complete loss of dihydroorotic dehydrogenase activity as measured by the aerobic assay. Although DOC treatment completely inactivated the dihydroorotic dehydrogenase activity as measured by the aerobic assay, 36% of the initial ferricyanide reduction activity remained (Table 5). Dihydroorotic dehydrogenase activity in the water control was approximately the same when measured by either the aerobic or ferricyanide assay. The enzyme activity present after DOC treatment was not sedimented by centrifugation at 100,000 × *g* for 2 hr. Repeated treatment of the soluble dihydroorotic dehydrogenase with DOC resulted in further enzyme inactivation. A separate experiment showed that the addition of DOC to the ferricyanide reaction mixture did not inhibit ferricyanide reduction. Thus, the loss of enzyme units was not due to the presence of DOC in the reaction mixture. Data on the removal of enzyme from washed particles and purification of the DOC-soluble enzyme by ammonium sulfate fractionation are in Table 6. Although some of the activity was destroyed by DOC, most of the remaining activity was soluble. The values given for the

ammonium sulfate fraction in Table 6 represent only the enzyme precipitated between 0.4 and 0.5 ammonium sulfate saturation. In spite of the loss in enzyme activity during DOC treatment, the enzyme was purified approximately tenfold from washed particles. This represents a 20-fold purification from whole-cell activity. Attempts to extend and improve this procedure are continuing.

DISCUSSION

Lieberman and Kornberg (1953) described an induced dihydroorotic dehydrogenase formed by an anaerobic bacterium, *Zymobacterium oroticum*, in response to growth on orotate. This enzyme was purified and subsequently crystallized by Friedmann and Vennesland (1958, 1960), and was reported to be a flavoprotein with two activities: oxidation of NADH by molecular oxygen and reduction of orotate to DHO by NADH. Reynolds, Lieberman, and Kornberg (1955) reported that the degradative dihydroorotic dehydrogenase of two isolates of aerobic bacteria, placed in the genus *Corynebacterium*, was linked to NADP rather than to NAD. A similar enzyme was recently purified by Udaka and Vennesland (1962). The biosynthetic enzyme in *E. coli* which we are studying does not appear to be linked to pyridine nucleotides. Thus, the biosynthetic enzyme seems to be different from the enzyme involved in degradation of orotate by other organisms. The DOC-soluble dihydroorotic dehydrogenase which we prepared does not react directly with oxygen, and reduction of ferricyanide by DHO is not affected by cyanide. We are now working on further purification and characterization of the biosynthetic enzyme. Our results are inconsistent with the data of Sheinen (1958), who studied enzymes in pyrimidine synthesis with the use of *E. coli* B and a histidine-uracil requiring mutant of this strain. Dihydroorotic dehydrogenase activity was measured by assay of the amount of DHO produced from orotate in the presence of sonic extract and NADH. No mention was made of NADH oxidation by orotate, and no evidence was given to show that the reaction product was DHO. The activity measured was present only in uracil-grown cells and only in one short phase of growth: the early lag phase for the wild-type and the early exponential phase for the mutant.

Yates and Pardee (1957) and Beckwith et al. (1962) measured the *E. coli* enzyme as an oxidase in intact cells and crude extracts. This is explained by our data showing that the enzyme is bound to an oxidative particle.

TABLE 6. *Removal of dihydroorotic dehydrogenase from particles and partial purification of the enzyme**

Fraction	Enzyme activity	
	Total units	Specific activity
Washed particles	7,940	7.6
Deoxycholate-treated particles	5,230	4.7
Supernatant fluid, 100,000 × *g*	4,410	8.8
Dialyzed supernatant	3,242	6.8
$(NH_4)_2SO_4$ precipitate	1,530	67.6

* Details of the purification procedure are given in Materials and Methods.

Because it has been possible to obtain pure membrane fractions from gram-positive bacteria through the use of lysozyme protoplasts, the localization of enzymes has been widely studied. Succinic, malic, lactic, and α-ketoglutaric dehydrogenases and NADH oxidase were present in the isolated membranes of *Bacillus megaterium*. These enzymes, as well as the bulk of the cytochromes, are found in the particulate fraction when cells are disrupted by grinding with abrasives or by sonic oscillation. In all organisms studied, except *Acetobacter*, most dehydrogenases which reduce pyridine nucleotides are soluble (Marr, 1960). Similar results were obtained with cell wall-membrane fractions of gram-negative bacteria (Hughes, 1962).

There is no effective method for preparation of pure membranes from gram-negative bacteria, and preparation of membranes containing no cell wall is further complicated because the cell wall and membrane of gram-negative bacteria are multilayered. However, Hughes (1962) showed that fractions containing cell wall-membrane particles are produced when cells are disrupted with a French press. All of the enzymatic activities associated with the membrane fraction of spheroplasts were found in the cell wall-membrane particles. This is in agreement with our finding that dihydroorotic dehydrogenase was present in the membrane fraction from spheroplasts and was localized on particles of French press extracts. There was no evidence given by Friedmann and Vennesland (1960) or Udaka and Vennesland (1962) concerning the localization of the degradative dihydroorotic dehydrogenase.

The removal of dihydroorotic dehydrogenase from membrane fractions by DOC implies that

the enzyme may be bound to a lipoprotein or a structure similar to submitochondrial particles (Green, 1962). This is further evidence that the enzyme is probably in the membrane of *E. coli* because most of the bacterial cell lipid is in the membrane (Hughes, 1962). Losses in enzyme activity are not usually encountered during fragmentation of mitochondria or bacterial membranes by bile salts (Green, 1962); however, Cooperstein (1963) reported that cytochrome oxidase from heart muscle was denatured by DOC treatment. Comparison of our DOC-soluble enzyme preparations with enzyme dissociated from particles when other agents are used may indicate the cause for losses in enzyme activity due to DOC treatment, and may provide clues regarding the nature of the enzyme in its natural state.

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