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AN ABSTRACT OF THE THESIS OF Pamela S. Gilchrist for the Master of Science in Biology presented August 6, 1975.

Title: The Role of Zinc in Dihydroorotase.

APPROVED BY MEMBERS OF THE THESIS COMMITTEE:

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Dihydroorotase (4,4-dihydroorotic acid amidolyase, EC 3.5.2.3.) which catalyzes the reversible cyclization of N-carbamyl-L-aspartate to L-dihydroorotate has been purified from orotate-grown *Clostridium oroticum*. The enzyme is stable in 0.3 M sodium chloride and 10 μM $ZnSO_4$. Sodium dodecyl sulfate gel electrophoresis indicates the enzyme to be composed of two identical subunits each with a molecular weight of 58,000 ± 6000. Dihydroorotase is shown to be a zinc-containing metalloenzyme with 2 g atoms of zinc per 58,000 g of protein. The role of zinc in dihydroorotase is discussed.

THE ROLE OF ZINC IN DIHYDROOROTASE

by

PAMELA S. GILCHRIST

A thesis submitted in partial fulfillment of the requirements for the degree of

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TO THE OFFICE OF GRADUATE STUDIES AND RESEARCH:

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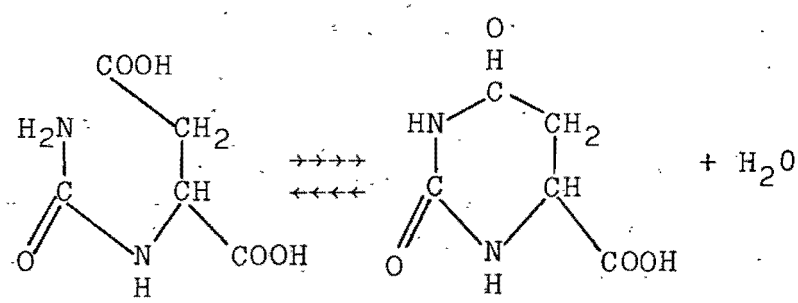
INTRODUCTION

Dihydroorotase (4,5-dihydroorotate amidolyase, EC 3.5.2.3) catalyzes the reversible cyclization of N-carbamyl-*L*-aspartate to *L*-dihydroorotate.



N-carbamyl-*L*-aspartate *L*-dihydroorotate

This reaction is intermediate in the biosynthesis and degradation of pyrimidines. The pathway for orotate catabolism was first demonstrated by Lieberman and Kornberg (11, 12, 13) who studied the orotate-fermenting bacterium *Clostridium oroticum*. This organism, isolated by Kornberg in Dr. H. A. Barker's laboratory, has been the source of most of the study on the degradation of pyrimidines.

Yates and Pardee (26) showed that dihydroorotate dehydrogenase was produced in large quantities when *Cl. oroticum* was cultured on media containing orotate carbon source, but not when glucose was the carbon source. Subsequently, they investigated the regulatory properties of pyrimidine biosynthesis in *E. coli B*. Their work has been expanded by Beckwith *et al.* (2) and Taylor *et al.* (20), who conclusively demonstrated that the enzymes for pyrimidine biosynthesis in *E. coli B* are under repression control.

Derepression of a mutant of *E. coli* K-12, which requires uracil for growth on minimal media, yielded increased quantities of dihydroorotate dehydrogenase. Characterization and partial purification of this enzyme by Taylor *et al.* (21, 22) suggested that the biosynthetic dihydroorotate dehydrogenase was significantly different from the enzyme produced when an organism is grown on orotate. Examination of the properties of both the biosynthetic and degradative dihydroorotate dehydrogenases from a pseudomonad isolated by Taylor *et al.* (22) conclusively showed that there were two distinct enzymes.

Although dihydroorotate dehydrogenase has been investigated extensively, very little attention has been focused on the remaining enzymes of the pathway. Among these enzymes is dihydroorotase. First described by Lieberman and Kornberg (12), it was partially purified from *Cl. oroticum* by Sander *et al.* (18). They were able to demonstrate a metal ion requirement (probably zinc) for enzymatic activity. Balch (1) purified the enzyme to homogeneity, and determined a mole ratio of zinc to protein of four, but did not study the role of zinc in enzymatic activity.

The present study was undertaken to purify dihydroorotase from extracts of *Cl. oroticum* in order to: 1) provide a stable enzyme with an improved yield, and 2) study the relationship of enzyme activity to metal ion requirements.

MATERIALS AND METHODS

Chemicals

All chemicals were obtained commercially and, unless otherwise stated, they were either analytical or reagent grade. The following were obtained from Sigma Chemical Company: streptomycin sulfate, tris(hydroxymethyl)-aminomethane (Tris), 2(N-morpholino)ethane sulfonic acid (MES), 1,10-phenanthroline (OP), N-carbamyl-*D,L*-aspartate [*D,L*-urcidosuccinate (*D,L*-US)], *L*-dihydroorotate (*L*-DHO), *D*-dihydroorotate (*D*-DHO), Coomassie Brilliant Blue R, 2-mercaptoethanol (2-hydroxymethyl-mercaptan), sodium dodecyl sulfate (SDS), ethylenediaminetetraacetic acid, disodium salt (EDTA), and sodium orotate. Sodium chloride was obtained from Mallinkrodt Chemical Co. Hydroxyapatite (Bio Gel HT) was purchased from Bio-Rad. Acrylamide, N,N'-methylenebisacrylamide, ammomium persulfate and N,N,N',N'-tetramethylenediamine (TMEDA) were Bio-Rad electrophoresis grade. Sephadex G-25-300 and DEAE Sephadex A-50 were obtained from Pharmacia. Diacetylmonoxime (2,3 butanedione Monoxime) and 1,5-dimethyl-2-phenyl-3-pyrazolone (antipyrine) were products of Matheson Coleman & Bell. Riboflavin was purchased from Mann Research Laboratories, Inc. Protein standards were obtained as follows: bovine serum albumin (Fraction V powder), Myoglobin (Type II), ovalbumin (Grade V), pepsin (2X crystallized and lypholized) from Sigma Chemical Co.; trypsin (TPCK) from Worthington and catalase and Blue Dextran 2000 from the Pharmacia calibration kit.

All reagent solutions were made with glass distilled water.

Enzyme Assays

Dihydroorotase activity could be measured in either the biosynthetic or degradative direction as outlined below. The biosynthetic conversion of *D,L*-US to *L*-DHO was measured using a modification of the method of Sander *et al.* (18). The reaction mixture contained 67 µmoles/ml MES-NaOH buffer (pH 6.0), 6.7 µmoles/ml *D,L*-US, and unless otherwise specified, 50 µl enzyme (approximately 1-2 units per ml). The reaction was initiated by the addition of substrate. Unless otherwise stated, the total volume of the reaction mixture was 1.5 ml. The enzymatic reaction was carried out at room temperature and at pH 6.0, the pH optimum for this reaction as determined by Balch (1). In contrast to the finding of Sander (18) incubation of enzyme with buffer prior to the initiation of the reaction with substrate was found not to be necessary. The linear increase in absorbance at 230 nm due to the formation of *L*-DHO was followed for at least three minutes using a Cary 14 recording spectrophotometer. The extinction coefficient for *L*-DHO of 1.17 $mM^{-1}cm^{-1}$ as reported by Sander (18) was used in all calculations.

A unit of dihydroorotase activity is defined as that quantity of enzyme which will catalyze the production of 1 mmole of *L*-DHO per minute under the conditions given for

the biosynthetic assay. Specific activity is listed as units of enzyme per milligram of protein and refers to activity when *D,L*-US is used as substrate.

The procedure of Prescott and Jones (17) was used to measure the quantity of *L*-US enzymatically formed from *L*-DHO with time. The enzyme reaction mixture contained 100 µmoles/ml Tris-acetate buffer (pH 8.0) and 1.2 µmoles/ml *L*-DHO. The enzymatic reaction was carried out at room temperature and pH 8.0, the pH optimum for this reaction as determined by Balch (1). The reaction was initiated by the addition of enzyme (50 µl, 1-2 units per ml). For termination of the enzymatic reaction, aliquots (0.1 ml) were removed at desired time intervals and diluted into mixtures containing 0.9 ml water and 1.0 ml antipyrine-oxime reagent. Standard curves were included in each run. The color was developed by the more sensitive method, Method II (17). All precautions pertaining to the light sensitivity of the color reagent were observed. Color intensity at 466 nm was measured using a Cary 14 recording spectrophotometer.

Saturating levels of substrates were used in all enzyme assays, and corrections were made for any activity not dependent on the addition of substrate.

Solutions of the sodium salt of *L*-US (0.1 M) were prepared by suspending the solid in water, followed by addition of 1 N NaOH to pH 6.0. It was necessary to keep the solution of *L*-US in an ice bath to prevent spontaneous

decarboxylation. The solution was brought to final volume with water, aliquots were dispensed into test tubes and stored at -20°C. Solutions of the sodium salt of *L*-DHO (0.1 M) were prepared by suspending the solid in water, followed by addition of 1 N NaOH to pH 6.0. The solution was brought to volume with water and stored at 4°C.

Culture Methods

Large quantities of *Clostridium oroticum* cells were obtained by culturing the organisms in 13 liter carboys at 30°C using the following medium: tryptone, 50 g; yeast extract, 5 g; sodium thioglycollate, 5 g; KH_2PO_4, 13.6 g; K_2HPO_4, 69.5 g; sodium orotate, 51.3 g; water, 10 liters. The final pH of the medium was 7.5. [Stock cultures were maintained in the same medium plus 0.5% agar.] Three liters of actively fermenting *Cl. oroticum* were inoculated into 10 liters of freshly autoclaved media and maintained under intermittent swirling until gas production ceased and the orotate was nearly depleted. Subsequently, 38 g sodium orotate per 13 liters was added in two portions and the cells were harvested after vigorous gassing had resumed and most of the orotate had disappeared. Cells were harvested at 4000 rpm using a CEPA continuous-flow centrifuge (Carl Padberg GMBH, 7630 Lahr/Schwarzwald) with a type K head. The flow rate was maintained at 400 ml/min. The cell pellet was washed with 250 ml 0.05 M potassium phosphate buffer (pH 7.0) or TZ buffer [0.025 M Tris-HCl buffer (pH 7.6),

10^{-5} M $ZnSO_4$] and centrifuged at 16,000 x g for 30 minutes at 4°C. The supernatant was discarded and the pellet stored at -20°C. Cell yield was approximately 38.5 g wet weight per 13 liters of media.

Preparation of Cell-free Extracts

Washed cells which had been stored at -20°C were suspended in 0.05 M potassium phosphate buffer (pH 7) or TZ buffer and disrupted using a French Pressure cell (American Instrument Co., Inc.) at 7000 psi. The crude extract was centrifuged for 90 minutes at 150,000 x g in a Beckman Model L Preparative Ultracentrifuge. The pellet was discarded and the supernatant was used as the starting material for subsequent enzyme purification.

Purficiation of Dihydroorotase

Solid streptomycin sulfate (2.5 g per 100 ml of supernatant) was added slowly to the 150,000 x g supernatant at 4°C with stirring. Stirring was continued for 15 minutes after the final addition of streptomycin sulfate. The precipitated nucleic acids were removed by centrifugation at 16,000 x g for 20 minutes at 4°C. The insoluble material was discarded and the clear extract (30 ml) was layered onto a Sephadex G-25 column (2.5 by 84 cm) at room temperature. The column had previously been equilibrated with TZ buffer. Protein was eluted from the column with the equilibrating buffer using a flow rate of 1 ml per minute. The

enzymatically active fractions were combined and solid sodium chloride was added to give a final concentration of 0.3 M.

After storage at 4°C overnight, the enzyme fraction was diluted with TZ buffer to give 0.15 M sodium chloride and adsorbed (at room temperature) onto a cake of DEAE Sephadex A-50 equilibrated with TZ buffer (5 g dry weight). The protein was eluted with a minimum amount of TZ buffer containing 1 M sodium chloride. The eluate was diluted with TZ buffer to less than 0.3 M sodium chloride and adsorbed onto a cake of DEAE Sephadex A-50 (5 g dry weight) equilibrated with TZ buffer. The cake was washed with approximately 200 ml TZ buffer containing 0.25 M sodium chloride. The enzyme was then eluted with a minimum volume of TZ buffer contining 0.35 M sodium chloride.

The protein recovered from DEAE (diluted to give 0.1 M sodium chloride) was absorbed onto an hydroxyapatite column (1.6 by 8 cm) at 4°C. The column was washed with 0.1 M potassium phosphate buffer (pH 6.8) until no protein, as measured at 220 nm, was found in the eluate. The adsorbed protein was eluted with a linear gradient developed in a LKB gradient mixer (LKB Instruments, Inc.) from 150 ml volumes of 0.1 M and 0.5 M potassium phosphate buffer (pH 6.8).

The column was eluted with a flow rate of 40 to 60 ml per hour. The gradient was followed by conductance

readings using a conductivity bridge (Model 31, Yellow Springs Instrument Co., Inc.). Protein elution was monitored by absorbance at 220 nm using 0.1 M potassium phosphate buffer (pH 6.8) as a blank. Dihydroorotase was found to elute between 0.1 and 0.13 M potassium phosphate buffer (pH 6.8). Active fractions were combined as soon as possible and applied to a Sephadex G-25 column (2.5 by 34 cm) previously equilibrated with TZ buffer at room temperature. The phosphate-free enzyme was collected with the void volume. Knowing that barium nitrate is slightly soluble in aqueous solutions, and also knowing that barium phosphate is completely insoluble, a sensitive spot test was developed to check for the presence of phosphate in enzyme preparations. A drop of saturated barium nitrate solution and a drop of the column eluate from fractions on the tailing side of the void volume were mixed in a spot plate. A positive reaction for the presence of phosphate was indicated by a white precipitate of barium phosphate. Using this spot test, we could insure that phosphate and the enzyme had been adequately separated. Protein fractions containing no phosphate were combined made 0.3 M sodium chloride and concentrated to approximately 10 ml using an Amicon ultrafiltration apparatus (Amicon Corp.) with a PM-10 membrane. The final solution was sterilized by filtration and stored in a sterile serum vial to prevent the growth of bacteria.

Preparation of Hydroxyapatite Columns

Approximately 8 cm of hydroxyapatite (20 ml of suspended crystals) was layered into a K16/30 Pharmacia column according to the method described by Bernardi (3). In addition to the described precautions of Bernardi, it was necessary to place the hydroxyapatite over a layer of Sephadex G-25 to prevent clogging of the nylon screen. The flow adaptor screen was protected similarly by a 1 to 2 cm layer of Sephadex G-25 placed between the hydroxyapatite and the screen. Prior to use the column was equilibrated with 0.01 M potassium phosphate buffer (pH 6.8).

Careful handling of the hydroxyapatite crystals provided consistently good flow rates. Storage of the hydroxyapatite suspension in a commercial refrigerator was found to be unsatisfactory due to vibrations which destroyed the crystal structure of the hydroxyapatite. Thus, for reproducible flow rates, the hydroxyapatite suspension was stored at 4°C under vibration-free conditions provided by a walk-in refrigerator.

Acrylamide Gel Electrophoresis

Analytical disc gel electrophoresis was carried out using a Hoefer Model EF301 electrophoresis apparatus (Hoefer Scientific Instruments, Inc.) maintained at 4°C by an external circulating water bath. The general procedures of Davis (5) were used throughout. The gel tubes were prepared by washing with detergent and soaking in sulfuric-nitric

acid (60:40, v/v) before final rinsings with water and coating with Photo-Flo. The upper and lower gel composition and the Tris buffer system were as described by Jovin *et al.* (9). Purity of the final enzyme preparation was determined in 7.5% polyacrylamide gels. Protein bands in the gels were located using Coomassie Brilliant Blue R as described by Fairbanks *et al.* (8). The final destaining step was performed using a Hoefer diffusion destainer (Hoefer Scientific Instruments, Inc.). Density tracings of protein profiles of the gels were obtained using a Chromoscan Mk. II (Joyce, Loebl & Co., Ltd.).

Subunit Analysis

Subunit analysis was performed using polyacrylamide gel electrophoresis in the presence of SDS. Composition and molecular weight of the subunit structure of dihydroorotase was determined by a modification of the procedure described by Weber and Osborn (25) as developed by Loehr (14). Protein standards and dihydroorotase were treated with the same concentration of SDS. Protein standards (2 mg/ml) were prepared and an aliquot (0.2 ml) of the protein was incubated with 0.5 ml of a buffered solution (0.04 M Tris-acetate (pH 8.0), 0.002 M EDTA, 1% SDS, 1% 2-mercaptoethanol, and 40% sucrose) for 15 minutes in a 50°C water bath. After incubation, the standards were diluted with water to 1.0 ml to give a protein concentration of 0.4 mg/ml. Dihydroorotase (0.1 mg/ml) was incubated with an

equal volume of the buffered solution. For gel electrophoresis, 20 µg of each standard or 10 µg of the dihydroorotase was applied to the gel. Polyacrylamide gels contained 8% acrylamide and 0.2% N,N'-methylenebisacrylamide in a solution containing 0.2 M Tris-acetate buffer (pH 8.0), 0.001 M EDTA, 0.5% SDS, 0.08% TMEDA, and 0.1% ammonium persulfate. Approximately 2 ml of the gel solution was necessary to fill a 12.8 by 0.6 cm gel tube. Immediately after dispensing the gel solution, a few drops of isobutanol were layered over the gel to provide a flat surface. After polymerization, the isobutanol was removed, the gel surface washed several times with water and finally rinsed with reservoir buffer. Upper and lower reservoir buffers contained 0.03 M Tris-acetate buffer (pH 8.0), 0.001 M EDTA, and 0.5% SDS. After filling the reservoirs, the samples were layered carefully under the buffer onto the surfaces of the gels, and 25 µl 0.01% Brom phenol blue in 20% sucrose was added. Electrophoresis was carried out at 5 mA per tube until the tracking dye was visible as a disc in each gel, at which time the current was raised to 8 mA per tube for the duration of the experiment. Protein bands in the gels were located using Coomassie Brilliant Blue R as described by Fairbanks *et al.* (8) and final destaining was performed in a Hoefer diffusion destainer (Hoefer Scientific Instruments, Inc.) for 24-48 hours. The molecular weight of the subunits of dihydroorotase was determined from a standard

curve prepared by plotting log molecular weight *versus* mobility of the protein standards relative to the tracking dye. The subunit molecular weight of the standards used was taken from the work by Weber and Osborn (25).

Protein Determination

Protein elution profiles from DEAE Sephadex chromatography, Sephadex G-25 gel filtration and hydroxyapatite chromatography were followed by monitoring absorbance at 220 nm. This procedure was extremely useful for detection of proteins in dilute solution. After removal of most flavoproteins with DEAE Sephadex chromatography, protein concentration was determined by the spectrometric method of Warburg and Christian (24). Protein concentration for determination of specific activity was measured by the use of the Folin phenol reagent as described by Lowry *et al.* (15) with bovine serum albumin as the standard. The interference of Tris buffer was corrected for by making the initial dilution of the standard (bovine serum albumin) into TZ buffer and by making samples and standards to 1.0 ml volume with TZ buffer.

Atomic Absorption Spectroscopy

The zinc concentration at each enzyme purification step was determined as follows: 0.1 ml sample was diluted to 1.0 ml and dialyzed overnight against 500 ml 0.025 M Tris-citrate buffer (pH 7.8) or 0.025 M Tris-HCl buffer

(pH 7.6) using a Crowe-Englander Micro-Dialyzer Model MD101 (Hoefer Scientific Instruments, Inc.). After dialysis the zinc content was measured at 2138Å using a Perkin-Elmer Atomic Absorption Spectrophotometer Model 305B. Zinc nitrate (Harleco, 15 mM) was used as the stock standard. Linear standard curves were obtained from 1 to 15 μM, each containing a final concentration of 0.025 M Tris-HCl buffer (pH 7.6). [Tris buffer did not interfere with zinc determinations.]

Prevention of Contamination by Zinc

Special precautions were taken to avoid contamination by zinc prior to experiments involving the determination of the mole ratio of zinc to protein. All glassware was washed in hot detergent, rinsed well and soaked in concentrated sulfuric-nitric acid (60:40, v/v) for several hours before final rinsings with glass distilled water. Acid cleaned glassware was stored in Saran Wrap (Dow Chemical Co.). Standard zinc solutions were stored in tightly closed polyethylene containers. All reagents and buffers were made with glass-distilled water. [The zinc concentration of all reagents and buffers was determined to be less than 0.3 μM.]

Inhibition Studies

Inhibition studies with 1,10-phenanthroline and EDTA were performed with either of the enzyme substrates, *L*-DHO

or *D,L*-US. When *L*-DHO was the substrate, enzyme assay mixtures contained 100 µmoles Tris-acetate buffer (pH 8.0), a given concentration of inhibitor and water to a final volume of 0.88 ml. [Enzyme (20 µl, 1.8 units per ml; specific activity 28.7) was added to the reaction mixture at stated time intervals. After the specified incubation time the enzymatic reaction was initiated with 1.2 µmoles *L*-DHO. The total reaction volume was 1.0 ml. The enzymatic reaction was allowed to proceed for 5 minutes at which time it was terminated by the addition of 1.0 ml antipyrine-oxime reagent.] A reagent blank mixture contained all components except the enzyme. The control mixture contained all components except inhibitor. The quantity of *L*-US produced was measured by the method of Prescott and Jones (17) as described under Enzyme Assays.

The enzymatic reaction mixtures which would test for substrate protection from the inhibitors contained 0.2 mmoles of Tris-acetate buffer (pH 8.0), 2.4 µmoles of *L*-DHO, and 4 µmoles of 1,10-phenanthroline or 0.6 mmoles EDTA. The reaction was initiated by the addition of 50 µl enzyme (1.8 units per ml, specific activity 28.7). The total volume was 2.0 ml. [At 5 minute intervals after the initiation of the reaction 0.1 ml aliquots were removed from each reaction mixture and added to 0.9 ml water and 1.0 freshly prepared antipyrine-oxime reagent to terminate the reaction. Color development and measurement of the product, *L*-US,

was performed as described previously.] A reagent blank mixture contained all components except the enzyme. The control reaction mixture contained all components except inhibitor.

The effect of EDTA on the conversion of *D,L*-US to *L*-DHO was studied using a modified standard reaction mixture. EDTA (5 µmoles per ml) was incubated with 67 µmoles/ml MES-NaOH buffer (pH 6.0), 50 µl enzyme (1.5 units per ml, specific activity 23.8) and water to 1.4 ml for a stated period of time. The enzymatic reaction was initiated by the addition of 10 µmoles *D,L*-US (total volume 1.5 ml). The control reaction mixture containing enzyme, buffer and water was incubated for the same period of time as the corresponding reaction mixture containing inhibitor.

Initial reaction rates were used to calculate the percentage of inhibition.

RESULTS

Preparation of Cell-Free Extracts

Extracts prepared from cell pellets which were washed, suspended and disrupted in TZ buffer contained more soluble protein than comparable treatment of cell pellets with potassium phosphate buffer (pH 7.0). Extracts prepared in TZ buffer consistently gave final preparations of dihydroorotase that contained unusually high quantities of impurities and were unstable. As shown in Table I, the dihydroorotase specific activity and yield from Preparation A (cells broken in TZ buffer) were lower than that from Preparation B (cells broken in potassium phosphate buffer) after elution from hydroxyapatite. Addition of manganese chloride effectively removed nucleic acids, but the enzyme purified from this preparation was not stable.

Enzyme Purification

Dihydroorotase is unstable in all concentrations of potassium phosphate buffer (pH 6.8). This made us devise methods for hydroxyapatite chromatography which eliminated prolonged contact of dihydroorotase with phosphate. We found it unnecessary to equilibrate the enzyme fraction with potassium phosphate buffer (pH 6.8) prior to application to the hydroxyapatite column. Sephadex G-25 columns were used to substitute TZ buffer for the potassium phosphate buffer following cell disruption and hydroxyapatite chromatography. Elution of the protein with TZ buffer

SUMMARY OF PURIFICATION AND ZINC ANALYSIS FOR DIHYDROOROTASE

Table 1

Purification step	Preparation A			Preparation B		
	+μmoles Zn / mg protein	Specific activity	Percent yield	+μmoles Zn / mg protein	Specific activity	Percent yield
150,000 x g supernatant	0.003	0.80	---	0.003	1.03	---
G-25 eluate	0.017	1.60	57.0	0.011	2.19	118.2
0.3 M NaCl DEAE eluate	0.009	1.20	31.2	0.013	2.15	45.9*
Hydroxyapatite eluate (phosphate-free)	0.026	21.70	26.9	0.031	31.73	70.4

\+ A sample (0.1 ml) from each purification step was diluted to 1 ml and dialyzed overnight against 500 ml buffer followed by analysis of zinc at 2138.6 A as described in Materials and Methods. Preparation A: 0.025 M Tris-citrate dialysis buffer (pH 7.8). Preparation B: 0.025 M Tris-HCl dialysis buffer (pH 7.6).

* This reduced value is presumably due to a dilution error which resulted in the buffer containing only 10^{-6}M $ZnSO_4$, ten-fold less than normal.

0.025 M Tris-HCl buffer (pH 7.6) provided superior separation of phosphate from the enzyme. Generally, a time period of four hours was required from the absorption of the proteins onto hydroxyapatite to the final elution of dihydroorotase from the Sephadex G-25 column. Dihydroorotase preparations thus obtained contained less than 20% impurities as determined by disc gel electrophoresis. As seen in Table I (Preparation B), 70.4% of the origianl activity was recovered in the hydroxyapatite eluate.

Table I also contains metal analyses obtained during purification of dihydroorotase from two separate preparations. The zinc content, expressed as μmoles of Zn^{2+} per mg protein, rises from 0.003 μmoles Zn^{2+} per mg protein found in the crude extracts, to 0.026 μmoles and 0.031 μmoles per mg protein in fractions following hydroxyapatite chromatography. As the extraneous proteins are removed, the specific activity increases, and likewise, as extraneous zinc is removed, the ratio of zinc to protein increases.

Stability

As shown in Table I, there was a drop in enzymatic activity at the DEAE step. In preparation B, the $ZnSO_4$ concentration had been lowered to 10^{-6} M at this step, and the activity was partially recovered by the inclusion of 5×10^{-5} M $ZnSO_4$ in the buffer after the hydroxyapatite step.

After storage for four months at 4°C, DEAE eluates containing 0.3 M sodium chloride, had the same enzymatic

activity as originally recorded. This stability was attributed at least in part to the sodium chloride present in such fractions. Attempts to stabilize the activity of dihydroorotase after final purification showed that, in addition to $ZnSO_4$ (10^{-5} M), the addition of sodium chloride to a final concentration of 0.3 M was necessary for stabilization of enzyme activity. In the presence of 0.3 M sodium chloride, and 10^{-5} M $ZnSO_4$, the enzyme activity was relatively stable for at least 2 months when stored at 4°C. In contrast, preparations of dihydroorotase in the phosphate eluting buffer of the hydroxyapatite column were found to be totally inactive within 24 hours.

Phosphate Sensitivity

Standard methods for the use of hydroxyapatite chromatography indicate that the sample should be applied in the initial eluting buffer. When enzyme fractions containing dihydroorotase were dialyzed overnight against 0.01 M potassium phosphate buffer (pH 6.8), a small precipitate was observed. Formation of this precipitate correlated with loss of protein and enzymatic activity such that a calculated specific activity for the precipitated protein was equal to the specific activity of the purified enzyme obtained after hydroxyapatite chromatography. The precipitated protein could not be dissolved by dialysis against 0.025 M Tris-HCl buffer (pH 7.6). If $ZnSO_4$ was included in the Tris-dialysis buffer, a larger precipitate was observed.

The precipitate was not dissolved by changes in pH and was dissolved only by addition of 1 N NaOH to the pellet obtained following centrifugation. This type of behavior suggests a formation of an insoluble zinc-phosphate-protein complex.

Since the enzyme was found to be unstable in phosphate buffer, an experiment was performed to check the effect of phosphate on the enzymatic conversion of *D,L*-US to *L*-DHO. As shown in Table II, when potassium phosphate buffer (pH 6.0) was substituted for MES-NaOH buffer (pH 6.0) concentrations of phosphate up to 100 μmoles/ml showed little effect on the rate of *L*-DHO formation. The addition of higher concentrations of phosphate immediately decreased the enzymatic activity.

Inhibitor Studies

Dihydroorotase catalyzes the reversible cyclization of *L*-US to *L*-DHO involving the formation or cleavage of an amide bond. Because of the requirement for the presence of zinc during purification, the sensitivity of dihydroorotase towards the metal chelators, 1,10-phenanthroline and EDTA, was tested. A comparison of the results in Tables III and IV shows that substrate partially protects dihydroorotase against the effects of OP and EDTA. For example, 0.002 M OP inhibited only 15.4% when substrate was present prior to the addition of enzyme, but as seen in Table III, when the enzyme was incubated with the inhibitor for 30 minutes

Table 2

The effect of potassium phosphate on the formation of L-dihydroorotate from N-carbamyl-D,L-aspartate

Phosphate (μmoles/ml) in assay	Percent activity
0	100
67	108
100	101
200	49
333	16

Reaction mixtures contained the specified amount of potassium phosphate buffer (pH 60), enzyme and water to a final volume of 1.5 ml. The control assay contained 67 μmoles/ml MES-NaOH buffer (pH 60). The reactions were initiated by the addition of 10 μmoles l,L-US and the information of the product, L-DHO was followed as described in Materials and Methods.

Table 3

The effect of 1,10-phenanthroline and ethylenadiaminetetraacetic acid on the formation of N-carbamyl-L-aspartate: incubation of enzyme with inhibitor

Treatment of enzyme	Inhibitor concentration (M)	Enzymatic activity (nmoles L-US formed ml^{-1} min^{-1})	Percent Inhibition
None	0	12.70	0
OP	0.002	5.99	52.8
OP	0.008	3.17	75.0
EDTA	0.005	10.29	18.9
EDTA	0.1	3.27	74.2

Enzyme (20 µl spedific activity, 28.7) was mixed with 0.1 mmoles Tris-acetate (pH 8.0), OP (2 µmoles or 8 µmoles) or EDTA (5 µmoles of 100 µmoles), and water to a final volume of 0.9 ml. These mixtures were incubated for 30 minutes prior to the addition of 1.2 µmoles L-DHO (total reaction volume 1.0 ml). After 5 minutes, the enzyme reaction was terminated by the addition of the color reagent as described in Materials and Methods. Mistures not treated with inhibitors were incubated under the same conditions as those treated with OP and EDTA.

Table 4

The effect of 1,10-phenanthroline and ethylenediaminetetraacetic acid on the formation of N-carbamyl-L-aspartate: no incubation of the enzyme with inhibitor.

Treatment of enzyme	Inhibitor concentration	Enzymatic activity (nmoles L-US formed ml^{-1} min^{-1})	Percent inhibition
None	0	39.7	0
OP	0.002	33.6	15.4
EDTA	0.3	24.7	37.2

Reaction mixtures contained 0.2 mmoles Tris-acetate (pH 8.0), 2.4 µmoles L-DHO, 50 µl enzyme (specific activity 28.7), and 4 µmoles OP or 0.6 mmoles EDTA in a total volume of 2 ml. After 5 minutes, 0.1 ml aliquots were removed and analyzed for L-US as described in Materials and Methods.
Mixtures not treated with inhibitor were incubated under the same conditions as those treated with OP and EDTA.

prior to the addition of substrate, the inhibition by 0.002 M OP was 52.8%. A similar effect was seen when EDTA was substituted for OP. When the enzyme was incubated with inhibitor prior to initiation of the reaction, the inhibition by 0.1 M EDTA was so great that measurement of significantly lower quantities of *L*-US predicted if enzyme were incubated with 0.3 M EDTA was limited by the sensitivity of the assay.

Figures 1 and 2 show the results of an experiment designed to determine if the interaction of enzyme and inhibitor was time dependent. Enzyme (50 µl, specific activity 128.7) was incubated with inhibitor [OP (2 µmoles or 8 µmoles) or EDTA (5 µmoles or 100 µmoles)] for the indicated time periods prior to the initiation of the enzymatic reaction by the addition of 1.2 µmoles *L*-DHO. The control reaction mixture contained all components except inhibitor. As seen in Figures 1 and 2, the effect of EDTA and OP on the formation of *L*-US was concentration-dependent. Although dramatically inhibited, the quantity of *L*-US produced in the presence of a given EDTA concentration remained constant regardless of incubation time. In the control and inhibited reaction mixtures, the quantity of *L*-US produced decreased with incubation time.

The absorbance of OP at 230 nm prohibited its use in inhibition studies on the enzymatic conversion of *D,L*-US to *L*-DHO. Although EDTA also absorbs at 230 nm, concentrations up to 0.005 M EDTA can be used. As seen in Table V, the

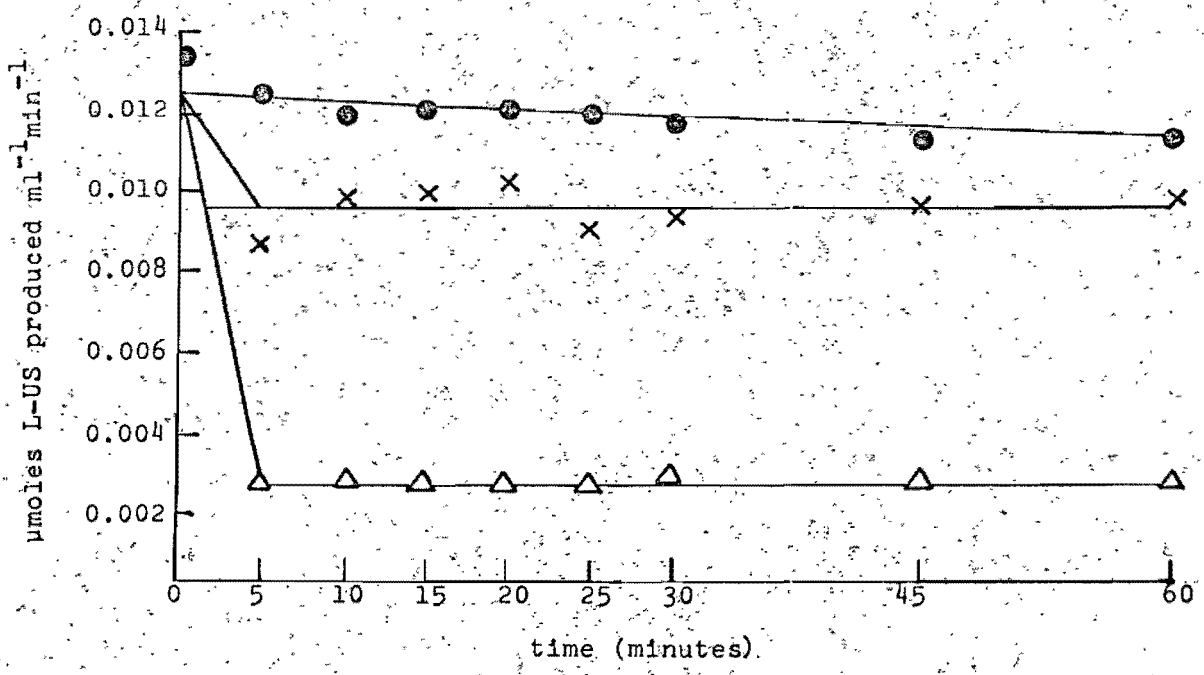


Figure 1. Effect of ethylenediaminetetraacetate on formation of N-carbamyl-L-aspartate from L-dihydroorotate

●——● control

X——X EDTA (0.005M)

Δ——Δ EDTA (0.1M)

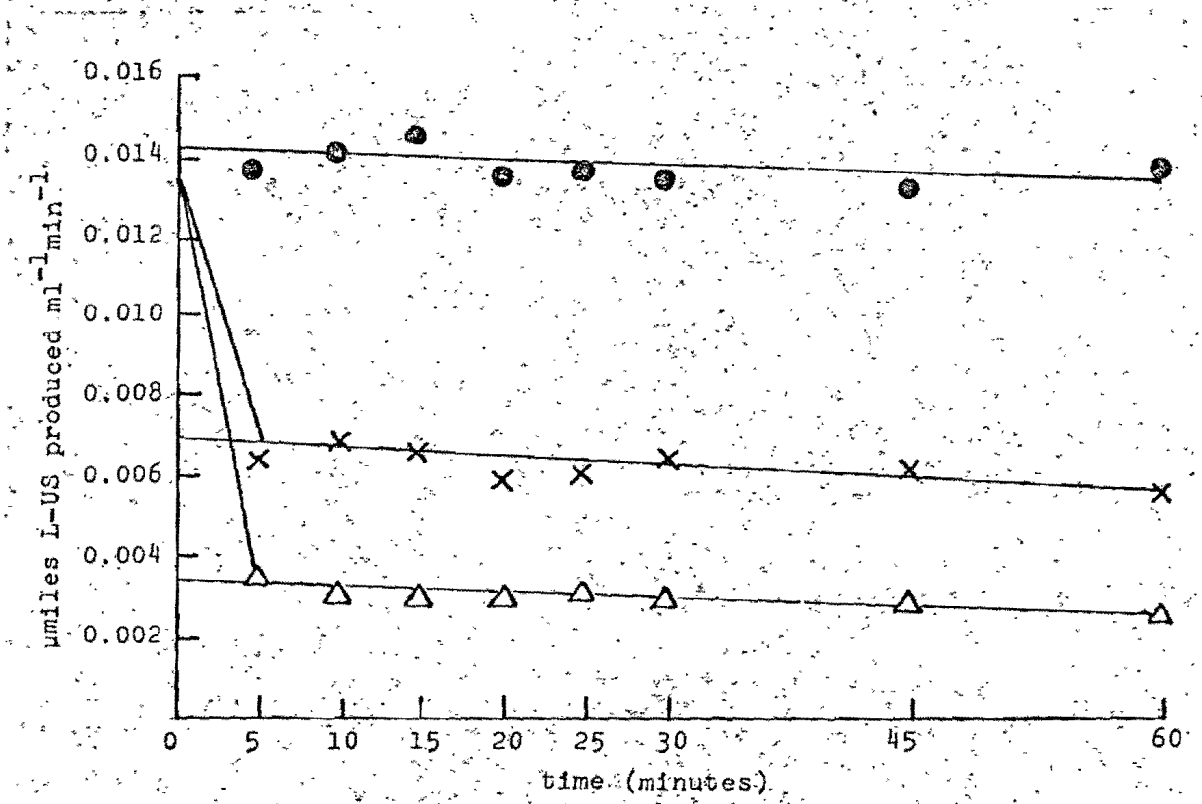


Figure 2. Effect of 1,10-phenanthroline on formation of N-carbamyl-L-aspartate from L-dihydroorotate

•——• control

X——X OP (0.002M)

Δ——Δ OP (0.008M)

Table 5

The effect of ethylenediaminetetraacetic acid on the formation of L-dihydroorotate from N-carbamyl-D,L-aspartate

Treatment	L-DHO produced μmoles ml^{-1} min^{-1}
None, no incubation	1.61
EDTA, no incubation	1.62
None, 30 min incubation	0.79
EDTA, 30 min incubation	1.01

The reaction mixtures contained 100 μmoles Mes-HuOH buffer (pH 6.0), 50 ul enzyme (specific activity, 28.7) and water. The inhibitor (or additional water in the control) was added (total volume, 1.4 ml) and the mixture incubated at room temperature for 30 minutes. After incubation, the enzymatic reaction was initiated by the addition of 10 μmoles D,L-US (total reaction volume 1.5 ml) and the formation of L-DHO was determined as described in Materials and Methods.

rate of formation of *L*-DHO from *D,L*-US, using the standard assay procedure as described in Materials and Methods, was the same in the presence or absence of 7.5 μmoles of EDTA.

In order to see if the effect of EDTA on the enzymatic conversion of *D,L*-US to *L*-DHO by dihydroorotase was time-dependent, the enzyme was incubated for 30 minutes in the presence and absence of inhibitor. As shown in Table V, dihydroorotase activity decays with time when maintained at pH 6.0. However, it would appear that the activity of the EDTA-treated enzyme did not decay as rapidly at this pH.

Although dilution effects must be considered, the decay of activity is most probably explained by the pH at which the incubation occurred. As seen in Figure 3, enzymatic activity decayed rapidly when dialyzed against buffer at pH 5.8, whereas dialysis against buffer at pH 8.0 showed a slower rate of enzymatic decay. Further evidence that this decay is pH dependent is shown in Figures 1 and 2 where control reactions at pH 8.0 exhibited only a slight decay of enzymatic activity over a period of 60 min. Loss of dihydroorotase activity at each pH tested was greater when the enzyme was dialyzed at the pH than when the pH of the enzyme was changed and the activity followed with time.

Zinc Determinations

In order to determine the mole ratio of zinc to protein, the zinc concentration of purified dihydroorotase was determined by two separate methods: 1) analysis for zinc

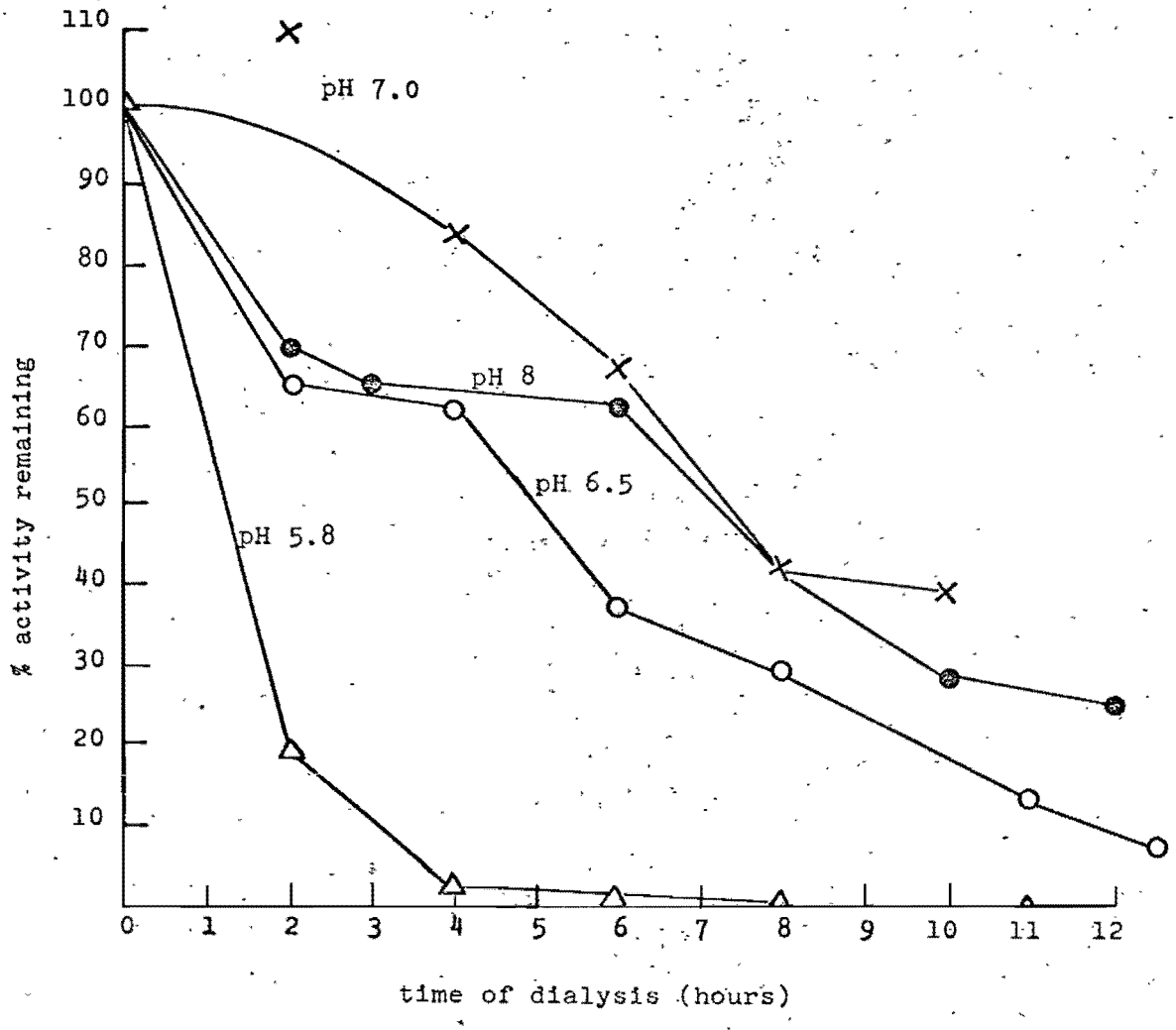


Figure 3. Effect of pH on stability of dihydroorotase

●—● pH 8 (0.1M Tris-HCℓ)

×—× pH 7 (0.1M Tris-acetate)

○—○ pH 6.5 (0.1M Tris-acetate)

△—△ pH 5.8 (0.1M Tris-acetate)

All buffers were 0.2M NaCl.

and correction for the known concentration of zinc in the storage buffer (TZ buffer), and 2) removal of extraneous zinc by dialysis of 1 ml of purified dihydroorotase against 500 ml 0.025 M Tris-citrate buffer (pH 7.8). These methods gave mole ratios of zinc to protein of 3.47 and 3.49 respectively. Although neither of the methods attempted are in themselves conclusive, the close correlation of the results by both methods indicates the presence of 4 zinc atoms in the active dihydroorotase after the actual protein concentrations were corrected for the 20% impurities present.

Attempts were made to correlate loss of enzymatic activity with concurrent loss of zinc by dialysis of the enzyme at pH values ranging from 5.8 to 8.0. Figure 2 shows the loss of enzymatic activity *versus* time of dialysis. As can be seen, the decay of activity was most rapid at pH 5.8, and least rapid probably between pH 7 and pH 8. However, we were unable to demonstrate a corresponding loss of zinc. After 12 hours dialysis there were still 4 zinc atoms per molecule of enzyme even though there was no enzymatic activity left.

Subunit Analysis

The number of subunits in the active dihydroorotase molecule had been determined by SDS polyacrylamide gel electrophoresis using enzyme preparations which had been stored in approximately 0.14 M potassium phosphate buffer,

pH 6.8 (1). Under these conditions only one subunit with a molecular weight of 56,000 was found. Because we had observed a zinc-phosphate-protein interaction, SDS-polyacrylamide gel electrophoresis was repeated using a phosphate-free dihydroorotase preparation in order to see if the 56,000 molecular weight subunit was an artifact due to a zinc-phosphate-dihydroorotase complex. No lower molecular weight species were found when phosphate-free dihydroorotase fractions were examined, and the subunit located was observed to have a molecular weight of 58,000 ± 6000.

DISCUSSION

It has been suspected for some time that a metal ion is required for dihydroorotase activity. Yates and Pardee (26) reported that an unknown cofactor was lost during purification of the enzyme from *C. oroticum*. Sander *et al.* (18) were able to reactivate EDTA-inactivated dihydroorotase by the addition of acid-, heat-, or ultraviolet-treated extracts, and additional experiments showed that lost activity could be restored by the addition of Zn^{2+} or Co^{2+}. However, Zn^{2+} was more effective at physiological concentrations. Dihydroorotase obtained from *Cl. oroticum* was purified to homogeneity by Balch (1), who reported 4 g atoms of zinc per mole of enzyme (molecular weight 110,000).

Results of this study also indicate the presence of 2 g atoms of zinc per subunit of dihydroorotase (subunit molecular weight 58,000). Metalloenzymes which require zinc for catalytic activity (such as carbonic anhydrase, carboxypeptidase, and alkaline phosphatase), usually contain 1 g atom of zinc per subunit. Assuming that the metal ion serves only a catalytic function and further assuming that there is one catalytic site per subunit, one would expect to find only one zinc per subunit. Sodium dodecyl sulfate gel electrophoresis of purified dihydroorotase gave evidence that the native enzyme is a dimer composed of two identical subunits each with a molecular weight of 58,000 ± 6000. It is possible, however, that SDS in the presence

of a reducing agent did not completely dissociate the enzyme and that dihydroorotase is actually a tetramer composed of four identical subunits each with one atom of zinc.

Alternatively, one could explain 2 zinc atoms per subunit by using horse liver alcohol dehydrogenase (LADH) as a model. Initial research with purified LADH indicated that the enzyme was a tetramer with one zinc atom per subunit (6). However, X-ray diffraction studies showed that LADH was a dimer with two zinc atoms per subunit (4). Differential reactivities of LADH towards various inhibitors, and two distinct rates of exchange of $^{65}Zn^{2+}$ for non-radioactive Zn^{2+}, indicate that, of the 2 zinc atoms per subunit, only one is involved in catalysis (7). Presumably, the two non-catalytic zinc atoms are involved in maintaining the three-dimensional structure of the enzyme. Certain aspects of this study of dihydroorotase also suggest that zinc may be required to maintain the structural integrity of the enzyme in addition to a role at the active site of the enzyme. Sander *et al.* (18) suggested that zinc might be involved in a structural role in dihydroorotase since it was necessary to incubate the enzyme with zinc prior to initiation of the enzyme reaction with substrate in order to obtain maximum initial velocity. We have been unable to find a stimulation by zinc in the reaction mixture, but we have found it necessary to include zinc at each purification step in order to maintain enzyme activity. Our study also provides the

following evidence that indicates the necessity for external zinc: 1) if the enzyme was eluted from DEAE-Sephadex in buffer containing ten-fold less zinc than usual, a dramatic loss in activity was noted, 2) although enzymatic activity decayed rapidly during dialysis against metal-free buffer, we were unable to demonstrate a concurrent loss of zinc, and 3) the requirement for zinc in the storage buffer is at concentrations greatly in excess of the concentration of the enzyme (twenty-fold excess of Zn^{2+}).

Taylor *et al.* (23) have demonstrated two distinct dihydroorotate dehydrogenase enzymes in the same bacterial preparation. One enzyme is constitutive, whereas the adaptive enzyme is formed only when the organism is grown with orotate as the carbon and energy source. The constitutive enzyme does not link to pyridine nucleotides and requires bound FMN for activity. In contrast, the adaptive enzyme from *Cl. oroticum* requires FMN and FAD for activity and links to pyridine nucleotides. It is tempting to suggest that an analogous situation exists with dihydroorotase: one enzyme that is constitutive and one that is inducible when the organism is grown on orotate. However, the evidence is unclear. A metal ion requirement has been demonstrated for the enzyme isolated from orotate-grown *Cl. oroticum*. Sander *et al.* (19), Mazus and Buchowicz (16) and Kennedy (10) have isolated constitutive dihydroorotase from various sources have not demonstrated metal requirement

associated with enzymatic activity. Unfortunately, atomic absorption spectroscopy or other analytical work to definitely determine the presence or absence of a metal ion in constitutive dihydroorotase preparations has not been done.

It is interesting to note that we were unable to show inhibition of dihydroorotase with EDTA when assayed in the biosynthetic direction, whereas the same concentration of EDTA definitely inhibited the enzymatic activity when assayed in the degradative direction. This could be explained if the zinc, which seems to be necessary for enzymatic activity, is involved in the binding of only one substrate, dihydroorotate with dihydroorotate as the substrate, a metal chelator would negatively effect only the rate of binding of dihydroorotate, and removal of the zinc may even enhance the rate of binding of US. Characterization of dihydroorotase, regardless of the source of the enzyme, has revealed K_m values which indicate a stronger affinity for *L*-DHO than *L*-US. If zinc is a prerequisite for the affinity of the enzyme for *L*-DHO, removal of the zinc may decrease the affinity for *L*-DHO and/or increase the affinity for *L*-US. This change in binding properties would be logical for an enzyme functioning as a biosynthetic enzyme; it would not be desirable to have a stronger affinity for the product than the substrate. Thus, even the stimulatory effect of phosphate, noted earlier, could be explained

since this effect was noticed when assays were performed in the biosynthetic direction. It is tempting to speculate that constitutive and inducible dihydroorotase are closely related; if zinc is present in the constitutive enzyme, it may serve only a structural function, whereas the zinc in the inducible enzyme probably is required in both catalytic and structural roles.

SUMMARY

1. Dihydroorotase (4,5-L-dihydroorotate amidolyase, EC 3.5.2.3.) has been purified from *Clostridium oroticum* cultured on orotate.
2. Subunit analysis show dihydroorotase to be composed of two identical subunits, each with a molecular weight of 58,000.
3. Atomic absorption spectroscopy of the enzyme show it to be a zinc containing metalloenzyme with 4 g atoms of zinc per molecule of dihydroorotase.
4. The possible catalytic and structural roles of zinc in dihydroorotase have been discussed.

LITERATURE CITED

1. Balch, William E. 1973. Purification and Characterization of Dihydroorotase from *Clostridium oroticum*, a zinc-containing metalloenzyme. M.S. Thesis. Portland State University.

2. Beckwith, J. R., A. B. Pardee, R. Austrian, and F. Jacob. 1962. Coordination of the Synthesis of the enzymes in the Pyrimidine Pathway of *Escherichia coli*. Journal of Molecular Biology 5:618-634.

3. Bernardi, G. 1971. Chromatography of Nucleic Acids on Hydroxyapatite Columns. In Methods in Enzymology (Grossman, L. and K. Moldave, eds.) Vol. XXI, Academic Press, N. Y., p. 95-102.

4. Bränden, C., H. Ecklund, B. Nordstrom, T. Boiwe, G. Söderlund, E. Zeppezauer, I. Ohlsson, and Å. Åkeson. 1973. Structure of Horse Liver Alcohol Dihydrogenase at 2.9Å Resolution. Proceedings of the National Academy of Science 70:2439-2442.

5. Davis, B. J. 1964. Disk electrophoresis. II. Method and application to human serum proteins. Annals of the New York Academy of Science. 121:404-427.

6. Drum, D. E., J. H. Harrison, T. K. Li, J. L. Bethune, B. L. Vallee. 1967. Structural and Functional Zinc in Horse Liver Alcohol Dehydrogenase. Proceedings of the Nation Academy of Sciences. 57:1434-1440.

7. Drum, D. E. 1970. Differential Chemical Reactivities of Zinc in Liver Alcohol Dehydrogenase. 9:4078-4086.

8. Fairbanks, G., T. L. Steck, and D. F. H. Wallach. 1971. Electrophoretic Analysis of the Major Polypeptides of the Human Erythrocyte Membrane. Biochemistry 10:2606-2617.

9. Jovin, T., A. Chrambach, and M. A. Naughton. 1964. An apparatus for preparative temperature-regulated polyacrylamide gel electrophoresis. Analytical Biochemistry 9:351-369.

10. Kennedy, J. 1974. Dihydroorotase from Rat Liver: Purification Properties and Regulatory Role in Pyrimidine Biosynthesis. Archives of Biochemistry and Biophysics. 160:358-365.

11. Lieberman, I., and A. Kornberg. 1953. Enzymic synthesis and breakdown of a Pyrimidine, Orotic Acid. I. Dihydroorotate Dehydrogenase. Biochemica Biophysica Acta. 12:223-234.

12. Lieberman, I., and A. Kornberg. 1954. Enzymic synthesis and breakdown of a Pyrimidine, Orotic Acid. II. Dihydroorotate, Ureidosuccinate, 5-Carboxymethylhydantoin. Journal of Biological Chemistry. 207:911-924.

13. Lieberman, I., and A. Kornberg. 1955. Enzymic synthesis and breakdown of a Pyrimidine, Orotic Acid. III. Ureidosuccinase. Journal of Biological Chemistry 212:909-919.

14. Loehr, J. Personal communication.

15. Lowry, O. H., M. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein Measurements with the Folin Phenol Reagent. Journal of Biological Chemistry 193:265-275.

16. Mazus, B., and J. Buchowicz. 1968. Purification and Properties of Dihydroorotase from Pea Plants. Acta Biochimica Polonica 15:318-325.

17. Prescott, L. M. and M. E. Jones. 1969. Modified Methods for the determination of Carbamyl Aspartate. Analytical Biochemistry 32:408-419.

18. Sander, E. G., L. D. Wright, and D. B. McCormick. 1965. Evidence for function of a Metal Ion in the Activity of Dihydroorotase from *Zymobacterium oroticum*. Journal of Biological Chemistry 240: 3628-3630.

19. Sander, E. G. and M. Heeb. 1971. Purification and Properties of Dihydroorotase from *Escherichia coli B*. Journal of Biological Chemistry 227: 442-452.

20. Taylor, A. L., J. R. Beckwith, A. B. Pardee, R. Austrian, and F. Jacob. 1964. The Chromosomal location of the structural gene for oroti orotidylic acid pyrophosphorylase in *Escherichia coli*. Journal of Molecular Biology 8:771.

21. Taylor, W. H. and G. D. Novelli. 1964. Enzymes of the Pyrimidine Pathway of *Escherichia coli*. I. Synthesis by Cells and Spheroplasts. Journal of Bacteriology 88:99-104.

22. Taylor, W. H. and M. L. Taylor. 1964. Enzymes of the Pyrimidine Pathway of *Escherichia coli*. II. Intracellular Localization and Characterization of Dihydroorotate Dehydrogenase. Journal of Bacteriology. 88:105-110.

23. Taylor, W. H., M. L. Taylor, and D. F. Eames. 1966. Two Functionally Different Dihydroorotate Dehydrogenases in Bacteria. Journal of Bacteriology. 91:2251-2256.

24. Warburg, O. and W. Christian. 1942. Isolierung und Kristallisation des Gärungsferments Enolase. Bioch. Z. 310:384-421.

25. Weber, K. and M. Osborn. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. Journal of Biological Chemistry. 244:4406-4412.

26. Yates, R. A. and A. B. Pardee. 1956. Pyrimidine Biosynthesis in *Escherichia coli*. Journal of Biological Chemistry. 221:743-756.