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Purification and Properties of Dihydroorotase, a Zinc-Containing Metalloenzyme in *Clostridium oroticum*

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Dihydroorotase (4,5-L-dihydro-orotate amidohydrolase [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 homogeneous when subjected to polyacrylamide gel electrophoresis and is stable at pH 7.6 in 0.3 M NaCl containing 10 μM $ZnSO_4$. The enzyme has a molecular weight of approximately 110,000. Sodium dodecyl sulfate gel electrophoresis, using three different buffer systems, indicated the enzyme is composed of two subunits, each having a molecular weight of 55,000. Dihydroorotase is shown by atomic absorption spectroscopy to be a zinc-containing metalloenzyme with 4 g-atoms of zinc per 110,000 g of protein. The pH optima for the conversion of *N*-carbamyl-L-aspartate to L-dihydroorotate and for L-dihydroorotate to *N*-carbamyl-L-aspartate are pH 6.0 and 8.2, respectively. The K_m values for *N*-carbamyl-L-aspartate and for L-dihydroorotate are 0.13 and 0.07 mM, respectively. Inhibitor studies indicate that zinc may be involved in the catalytic activity of the enzyme.

Dihydroorotase (4,5-L-dihydro-orotate amidohydrolase [EC 3.5.2.3]) catalyzes the reversible cyclization of *N*-carbamyl-L-aspartate to L-dihydroorotate as follows: *N*-carbamyl-L-aspartate $\rightleftharpoons$ L-dihydroorotate + H_2O. This reaction is intermediate in both the biosynthesis and degradation of pyrimidines. Lieberman and Kornberg (13) first demonstrated dihydroorotase activity and described the pyrimidine pathway in the orotate-fermenting bacterium *Zymobacterium oroticum* (now called *Clostridium oroticum* [4]), and Reynolds et al. (20) demonstrated the pathway in aerobic bacteria. Bresnick and Blatchford (3) partially characterized dihydroorotase from a mammalian system.

The degradative dihydroorotase from *C. oroticum* was partially purified by Sander et al. (22), and indirect evidence suggested that the enzyme required zinc for activity (18, 22). Beckwith et al. (1) reported that the biosynthetic dihydroorotase activity present in *Escherichia coli* was stimulated by zinc, but Sander and Heeb (21) were unable to demonstrate a zinc requirement with their purified preparation from *E. coli*.

Yates and Pardee (29) were able to measure dihydroorotase activity in extracts of *C. oroticum* prepared from orotate-grown cells but reported much lower activity in extracts prepared from glucose-grown cells. This anaerobic bacterium synthesizes the usual enzymes for pyrimidine biosynthesis under all growth conditions and is able to form new, and possibly different, enzymes for pyrimidine degradation when orotate is added to the culture medium. Thus, comparison of enzymes catalyzing the same reactions of the degradative and biosynthetic pathways is possible in this organism and should provide some insight into the evolution and regulatory properties of these enzymes.

The present study describes the purification and properties of the degradative dihydroorotase from *C. oroticum*. The role of this enzyme in pyrimidine degradation and biosynthesis is discussed. The enzyme is shown by direct analysis to be a zinc metalloprotein containing 4 g-atoms of zinc per mol of enzyme. The enzyme appears to be a dimer containing protein subunits of a molecular weight of approximately 55,000. A preliminary report of this work has appeared (W. H. Taylor, M. L. Taylor, and W. E. Balch, Abstr. Annu. Meet. Am. Soc. Microbiol. 1974, P12, p. 146).

MATERIALS AND METHODS

Culture methods. *C. oroticum* was cultured in a medium containing (grams per liter): tryptone, 5; yeast extract, 0.5; sodium thioglycolate, 0.5; potassium dihydrogen phosphate, 1.36; dipotassium phosphate, 6.95; sodium orotate, 5.13; and riboflavin, 0.015. Stock cultures were maintained in the above medium plus 0.5% agar and transferred into freshly steamed medium weekly. All cultures were incubated at 30°C. Large quantities of cells were obtained as follows: 20 ml of stock culture was inocu-

lated into 140 ml of freshly autoclaved medium and incubated until the culture was gassing actively; the culture was then transferred into 3 liters of freshly autoclaved medium in a 3-liter flask; when this culture was actively gassing, it was transferred into a 13-liter bottle containing 10 liters of medium and incubated with intermittent stirring to suspend undissolved orotate crystals; when gassing had ceased, 38 g of sodium orotate was added and the cells were harvested when most of the solid orotate had dissolved and the culture was still gassing. Cells were harvested using a CEPA continuous-flow centrifuge (Carl Padberg GBMH). The cell pellet was washed with 250 ml of 0.05 M potassium phosphate buffer (pH 7.0) and centrifuged (16,000 × *g*) for 30 min at 4°C. The supernatant fluid was discarded and the cell pellet was stored at −20°C. The cell yield was approximately 38.5 g (wet weight)/13 liters of medium.

Chemicals. All chemicals were obtained commercially, and unless otherwise stated they were analytical or reagent grade. The following were obtained from Sigma Chemical Co.: 2(*N*-morpholino)ethane sulfonic acid (MES), 1,10-phenanthroline (OP), *N*-carbamyl-DL-aspartate (DL-ureidosuccinate), L-dihydroorotate, D-dihydroorotate, and sodium orotate. Electrophoresis reagents, hydroxyapatite (Bio-Gel HT), and Chelex 100 were purchased from Bio-Rad Laboratories. Antipyrine (1,5-dimethyl-2-phenyl-3-pyrazolone) and diacetylmonoxime (2, 3-butanedione) were products of Matheson, Coleman and Bell.

Protein standards were purchased from Sigma Chemical Co., except trypsin (TRTPCK) was obtained from Worthington and aldolase and blue dextran were from the Pharmacia calibration kit. *N*-carbamyl-L-aspartate (L-ureidosuccinate) was synthesized by the method of Nyc and Mitchell (17). The product was separated by the procedure of Lieberman and Kornberg (13).

Preparation of cell-free extracts. Washed cells were suspended to approximately 0.3 g (wet cells)/ml in 0.05 M sodium phosphate buffer (pH 7.0) and disrupted with a French press (American Instrument Co., Inc.) at 7,000 lb of pressure per in^2. The crude extract was centrifuged at 0°C for 90 min at 150,000 × *g*. The pellets were discarded and the supernatant fluid was used as the starting material for enzyme purification.

Purification of dihydroorotase. Solid streptomycin sulfate (2.5 g/100 ml of supernatant fluid) was added slowly to the 150,000 × *g* supernatant fluid at 4°C with stirring. Stirring was continued for 15 min after the final addition of streptomycin sulfate. The precipitated nucleic acids were removed by centrifugation at 30,000 × *g* for 20 min at 4°C.

The clear extract (30 ml) was layered onto a Sephadex G-25 column (2.5 by 84 cm) equilibrated with 0.025 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.6) containing 10 μM $ZnSO_4$ (TZ buffer). Protein was eluted from the column at room temperature, and solid NaCl was added to the enzymatically active fractions to give a final concentration of 0.15 M.

After storage at 4°C overnight, the enzyme fraction was adsorbed (at room temperature) onto a cake of diethylaminoethyl (DEAE)-Sephadex A-50 (5 g, dry weight). The enzyme protein was eluted with a minimum amount of TZ buffer containing 1 M NaCl (approximately 120 ml).

The eluate was diluted with TZ buffer to less than 0.3 M NaCl and adsorbed onto a cake of DEAE-Sephadex A-50 (5 g, dry weight) equilibrated with TZ buffer containing 0.25 M NaCl. The enzyme protein was then eluted with a minimum volume of TZ buffer containing 0.35 M NaCl (approximately 75 ml).

The protein recovered from DEAE (diluted to give 0.2 M NaCl) was adsorbed onto a hydroxyapatite column (1.6 by 8 cm) at 4°C. The column was washed with 0.1 M potassium phosphate buffer (pH 6.8). The adsorbed protein was eluted with a linear gradient developed from 150-ml volumes each of 0.1 and 0.5 M potassium phosphate buffer (pH 6.8). Dihydroorotase was found to elute between 0.1 and 0.13 M potassium phosphate buffer (pH 6.8). Active fractions were applied immediately to a Sephadex G-25 column (2.5 by 34 cm) equilibrated with TZ buffer at room temperature. Fractions were tested for the presence of phosphate by the addition of a solution of barium nitrate. Protein fractions containing no phosphate were combined, adjusted to 0.3 M NaCl, and concentrated by an ultrafiltration apparatus (Amicon Corp.). The final solution was sterilized by filtration and stored in a sterile serum vial.

Enzyme assays. The biosynthetic conversion of L-ureidosuccinate to L-dihydroorotate was measured, using a modification of the direct assay of Sander et al. (22). The reaction mixture contained 67 μmol of MES-NaOH buffer (pH 6.0) per ml, 6.7 μmol of DL-ureidosuccinate per ml, and unless otherwise specified, 50 μl of enzyme (approximately 1 to 2 U/ml) in a total volume of 3.0 ml. The reaction was initiated by the addition of substrate. The linear increase in absorbance at 230 nm due to the formation of L-dihydroorotate (ϵ_{230} = 1.17 mM^{-1} cm^{-1}) (22) was followed with a Cary 14 recording spectrophotometer.

Assay of the degradative activity was performed by measuring the rate of L-ureidosuccinate formation from L-dihydroorotate. The reaction mixture contained 100 μmol of Tris-acetate buffer (pH 8.0) per ml and 1.2 μmol of L-dihydroorotate per ml. The reaction was initiated by the addition of enzyme (20 μl, 1 to 2 U/ml). Unless otherwise stated, the total volume of the reaction mixture was 1.0 ml. For termination of the enzymatic reaction, samples (0.1 ml) were diluted into 0.9 ml of water and 1.0 ml of antipyrine-oxime reagent. The color was developed by the procedure of Prescott and Jones (19), and the absorbance at 466 nm was measured using a Cary 14 recording spectrophotometer.

A unit of dihydroorotase activity is defined as the quantity of enzyme that will catalyze the production of 1 μmol of L-dihydroorotate per min 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-ureidosuccinate is used as substrate.

Protein determination. Protein concentration

was measured by use of the Folin phenol reagent (14) with bovine serum albumin as the standard. The interference by Tris buffer was corrected for by diluting samples and standards with TZ buffer.

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 useful for detection of proteins in dilute solution.

Polyacrylamide gel electrophoresis. Analytical polyacrylamide disc electrophoresis was carried out using a Hoefer model EF 301 electrophoresis apparatus (Hoefer Scientific Instruments, Inc.) maintained at 4°C. The general procedures of Davis (6) were used throughout with 7.5 and 12% polyacrylamide gels. The upper and lower gel composition and the Tris buffer system were as described by Jovin et al. (11). Gels were stained and destained by the procedure of Chrambach et al. (5) or as described by Fairbanks et al. (9) and stored in 7.5% acetic acid.

Dihydroorotase activity in polyacrylamide gel columns was detected after slicing the gel into 2-mm segments; each segment was incubated in the degradative assay mixture, and the amount of L-ureidosuccinate formed was measured.

Subunit analysis. Subunit analysis was carried out using polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) by the procedure of Shapiro et al. (23) as outlined by Weber and Osborn (28) or by the procedure of Neville (16), using a discontinuous buffer system. Gel composition, using the notation of Hjerten (10), was 11.1 × 0.9 when the Neville procedure was used and 11.4 × 0.25 when the procedure of Weber and Osborn was used. Protein bands in the gels were located using Coomassie brilliant blue R as described by Fairbanks et al. (9) or by the method of Weber and Osborn (28). Destaining was carried out for 24 to 48 h with a diffusion destainer (Hoefer Scientific Instruments, Inc.). The molecular weights of the standards were taken from the method of Weber and Osborn (28).

Molecular weight estimation. The molecular weight of dihydroorotase was determined by thin-layer gel filtration using a Pharmacia thin-layer gel apparatus. A 20-cm plate spread with a 0.6-mm layer of Sephadex G-200 was equilibrated with 0.2 M potassium phosphate buffer (pH 6.8) containing 50 μM $ZnSO_4$. Protein standards and purified enzyme (approximately 50 μg) were placed on the plate, and the plate angle was adjusted to 20°, producing a flow rate of 3 cm/h for the excluded thyroglobulin. Enzyme was detected by transferring sections (0.5 by 2 cm) of the gel to the standard degradative assay mixture and detecting the formation of L-ureidosuccinate by the procedure of Prescott and Jones (19). The gel section giving the maximum amount of L-ureidosuccinate formation was considered to be the center of the dihydroorotase spot. Molecular weight markers were detected by absorbing them onto filter paper (Whatman 3 MM) and staining with 0.25% Coomassie brilliant blue R in methanol-acetic acid (90:10, vol/vol) for 15 min. The paper was destained by a 5-min wash in cold tap water, followed by a 12-h soak in ethanol-acetic acid-water (50:10:50, vol/vol/vol). Inverse migration distance (relative to thyroglobulin) was plotted versus log molecular weight.

Atomic absorption spectroscopy. Purified dihydroorotase was concentrated to approximately 0.75 mg/ml by ultrafiltration, and unbound zinc present in the storage buffer was removed by passage through a Sephadex G-25 column (1.6 by 19 cm) equilibrated with 0.025 M Tris-hydrochloride buffer (pH 7.8) or by dialysis of 1 ml against 500 ml of 0.025 M Tris-citrate buffer (pH 7.8) or 0.025 M Tris-hydrochloride buffer (pH 7.6) using a Crowe-Englander Micro-Dialyzer model MD101 (Hoefer Scientific Instruments, Inc.). Samples were analyzed for zinc content at 213.8 nm using a Perkin-Elmer atomic absorption spectrophotometer model 305B. A zinc nitrate solution (Harleco) was used as the standard.

Inhibitor studies. The effect of inhibitors on the conversion of L-dihydroorotate to L-ureidosuccinate was studied using the assay mixture described above with the following exceptions. (i) When the reaction was to be initiated by substrate, the reaction mixture containing OP or ethylenediaminetetraacetic acid (EDTA) was incubated for 5 to 30 min at room temperature prior to the addition of substrate. Five minutes after the addition of substrate, the reaction was terminated by the addition of antipyrine-oxime reagent. (ii) When the reaction was to be initiated by enzyme, the assay mixture contained 2 μmol of L-dihydroorotate and OP or EDTA. Portions were terminated at 5-min intervals by dilution into antipyrine-oxime reagent.

The effect of EDTA on the conversion of L-ureidosuccinate to L-dihydroorotate was studied by the inclusion of EDTA (up to 5 mM) in the assay mixture described above. Inhibition by OP could not be studied because of the high extinction coefficient at 230 nm.

RESULTS

Purification of dihydroorotase. The results of the procedure used for purification of dihydroorotase from *C. oroticum* are shown in Table 1. This procedure provides a 40-fold purification of dihydroorotase with a high (70%) recovery of enzyme units. Since we found it unnecessary to equilibrate the protein fraction with 0.01 M potassium phosphate buffer (pH 6.8) prior to application on hydroxyapatite, only a 4- to 5-h time period was necessary from adsorption of dihydroorotase onto hydroxyapatite to elution of the phosphate-free protein from Sephadex G-25. Enzyme eluted from hydroxyapatite and stored in the phosphate-eluting buffer was stimulated two- to threefold by incubation in 50 μM zinc sulfate. In contrast, enzyme eluted from hydroxyapatite followed by immediate change of the phosphate-eluting buffer to Tris buffer with Sephadex G-25 was not stimulated by incubation in 50 μM zinc sulfate. Also, this preparation was not stimulated by addition of zinc to the enzyme assay mixture. However,

TABLE 1. *Purification of dihydroorotase from C. oroticum*

Fraction	Total units	Total protein (mg)	Sp act (U/mg)
Supernatant fluid (150,000 × *g*, 90 min)	159	198	0.80
Sephadex G-25 eluate	188	168	1.12
DEAE-Sephadex eluate, 0.35 M NaCl	74	33.6	2.26
Hydroxyapatite eluate (pH 6.8)	112	3.5	32.00

the presence of zinc during purification was found to be essential as the recovery of enzyme units was only 15 to 20% if the procedure was performed in the absence of added zinc. The phosphate-free dihydroorotase prepared by the procedure outlined in Table 1 is stable at 4°C for up to 3 months in TZ buffer containing 0.3 M NaCl. However, enzymatic activity was lost when the preparations were stored at −20°C. This enzyme has remarkable stability at room temperature: eluates from DEAE-Sephadex retained all enzymatic activity during storage at room temperature for 3 weeks.

During the development of the purification procedure, we observed that the migration distance of dihydroorotase on 7.5% polyacrylamide gels almost coincided with that of the purified dihydroorotate dehydrogenase obtained from the same organism. The almost identical behavior in polyacrylamide gels must reflect a similarity in overall charge, since the molecular weights of these enzymes are almost identical (110,000 for dihydroorotase and 115,000 for the dihydroorotate dehydrogenase [W. H. Taylor, D. F. Eames, and M. L. Taylor, Abstr. Annu. Meet. Am. Soc. Microbiol. 1972, P168, p. 164]). The following evidence indicates that our dihydroorotase preparations were free from the dehydrogenase and other protein contaminants: (i) no dehydrogenase activity or flavin content was observed; (ii) treatment with SDS and subsequent electrophoresis in SDS-polyacrylamide gels revealed subunits corresponding to the dihydroorotase but no protein subunits that would have been present in the dehydrogenase; and (iii) electrophoresis on 7.5 and 12% polyacrylamide gels revealed only one protein band. Figure 1 shows the coincidence between the protein band and enzyme activity obtained by slicing a 7.5% gel longitudinally down its axis.

Only the L-isomers of dihydroorotate and ureidosuccinate are the substrates for dihydroorotase. The presence of D-ureidosuccinate does not inhibit dihydroorotase since equal rates of L-dihydroorotate were measured when either L-ureidosuccinate or racemic DL-ureidosuccinate was used as the substrate. In contrast, we observed a 32% inhibition of L-ureidosuccinate formation by dihydroorotase when D-dihydroorotate and L-dihydroorotate were present in equal concentration.

Effect of phosphate on enzyme activity. Since early experiments had shown the enzyme to be unstable when stored in phosphate buffer, the effect of phosphate on the enzymatic conversion of L-ureidosuccinate to L-dihydroorotate was examined. When potassium phosphate buffer (pH 6.0) was substituted for MES-NaOH buffer (pH 6.0), concentrations of phosphate up to 100 mM showed little effect on the rate of L-dihydroorotate formation. However, the addition of higher concentrations of phosphate markedly inhibited the enzymatic activity: 200 mM phosphate caused 51% inhibition and 300 mM phosphate caused 84% inhibition. Separate experiments using Na_2SO_4 and NaCl demonstrated that phosphate inhibition at high concentrations was not due to an ionic strength effect on the enzyme. When dihydroorotase in Tris or TZ buffer was dialyzed overnight against 10 mM potassium phosphate buffer, pH 6.8, a precipitate formed. The amount of protein and the number of enzyme units lost during dialysis indicated that the precipitate was a zinc-phosphate-protein complex. Thus, the effect of phosphate on dihydroorotase activity may be due to chelation of zinc near the active site.

Determination of pH optima. The pH optima for dihydroorotase for both substrates (L-ureidosuccinate and L-dihydroorotate) were determined. As shown in Fig. 2, the formation of L-dihydroorotate from L-ureidosuccinate shows a narrow pH optimum range from pH 5.8 to 6.2. Dihydroorotase activity in the biosynthetic direction is negligible below pH 5.5 or above pH 7.8.

Figure 3 shows that the pH dependence for conversion of L-dihydroorotate to L-ureidosuccinate is distinctly different from the reverse reaction and shows a broad pH optimum from pH 7.5 to 9.0. Dihydroorotase is quite active at pH 10 and is stable for weeks at this pH value. Use of Tris-hydrochloride buffers instead of Tris-phosphate or Tris-acetate buffers in the degradative assay generally resulted in 25 to 30% less activity. Although the degradative reaction exhibits a wider pH range of activity than does the biosynthetic reaction, the differ-

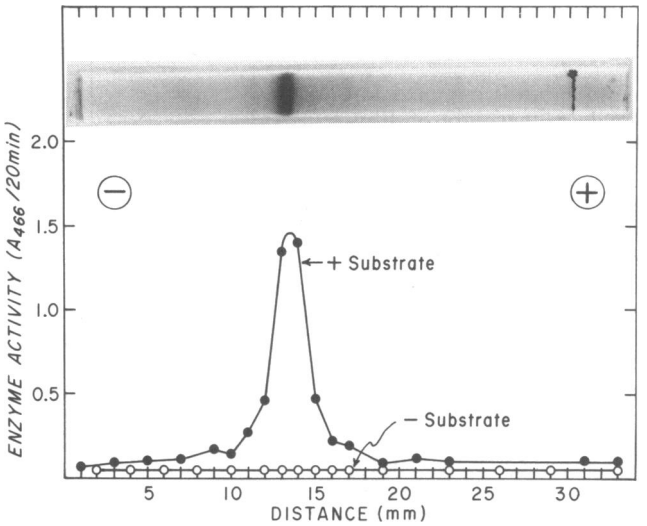

FIG. 1. *Comparison of protein staining and enzymatic activity assays on a polyacrylamide gel column. Approximately 50 μg (specific activity, 21) of enzyme protein was applied to the gel column. One-half of the gel was stained for protein and the other half was split into 2-mm sections, and each section was assayed for activity with L-dihydroorotate as the substrate. A duplicate gel was incubated without substrate as a control.*

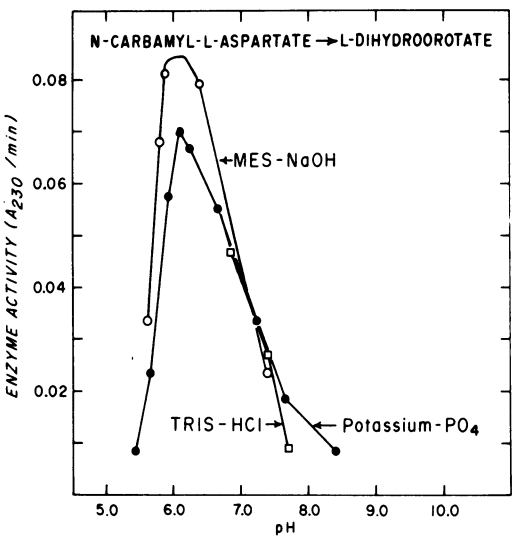

FIG. 2. *Effect of pH on dihydroorotase-catalyzed conversion of N-carbamyl-L-aspartate to L-dihydroorotate. The reaction conditions are stated in the text, using 0.063 U of enzyme (specific activity, 21). Buffers: 67 mM MES-NaOH (○), 167 mM potassium phosphate (●), 167 mM Tris-hydrochloride (□).*

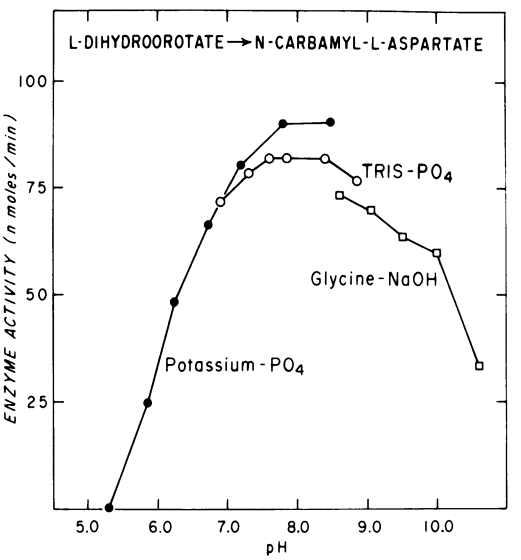

FIG. 3. *Effect of pH on dihydroorotase-catalyzed conversion of L-dihydroorotate to N-carbamyl-L-aspartate. The reaction conditions are listed in the text, using 0.031 U of enzyme (specific activity, 21). Buffers: 167 mM Tris-phosphate (○), 167 mM potassium phosphate (●), 167 mM glycine-NaOH (□).*

ences in the centers of optimal activity (pH 6.0 for the biosynthetic reaction and pH 8.2 for the degradative reaction) make the competing reactions appear to be mutually exclusive of one another.

Influence of substrate concentration. The K_m values for L-dihydroorotate (0.7 mM) and L-ureidosuccinate (0.13 mM) were extrapolated from Lineweaver-Burk plots. High substrate concentrations did not inhibit dihydroorotase activity of stable enzyme preparations. In contrast, the enzymatic activity in both the degradative and biosynthetic directions was in-

hibited by high substrate concentrations in unstable fractions, i.e., those stored in phosphate buffer.

Molecular weight and subunit composition of the enzyme. The molecular weight of dihydroorotase estimated by thin-layer gel filtration is approximately 110,000 (Fig. 4). Native dihydroorotase was analyzed for subunit composition in SDS-polyacrylamide gels by the procedures of Weber and Osborn (28) and Neville (16). As shown in Fig. 5, a monomer molecular weight of approximately 55,000 was obtained in the Weber and Osborn system. The same value was obtained in the Neville system. Further dissociation of the subunits was attempted by a modification of the Weber and Osborn procedure: prior to electrophoresis, the native protein was treated at pH 8.0 and 50°C with EDTA (1.4 mM), SDS (0.07%), and 2-mercaptoethanol (0.07%). Electrophoresis of this preparation again revealed a monomer with a molecular weight of 55,000 and no evidence of a smaller subunit protein. Thus, the dihydroorotase protein from *C. oroticum* is probably composed of two subunits, each having a molecular weight of 55,000.

Analysis for zinc. Evidence that dihydroorotase is a metalloprotein is the increase in ratio

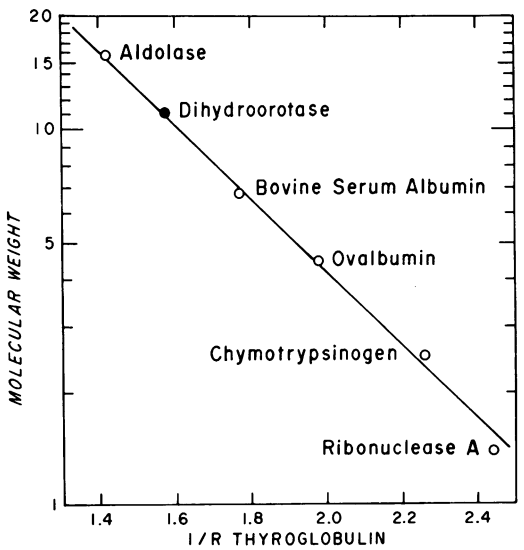

FIG. 4. *Estimation of the molecular weight of dihydroorotase using thin-layer gel filtration. The procedure is outlined in the text, using approximately 0.1 U of enzyme (specific activity, 21) on a 20-cm thin-layer gel plate coated with Sephadex G-200 superfine (0.6 mm). Molecular weight markers: aldolase, 158,000; bovine serum albumin, 68,000; ovalbumin, 45,000; chymotrypsinogen, 25,000; ribonuclease A, 13,700. The molecular weights are expressed in units of 10^4 g/mol.*

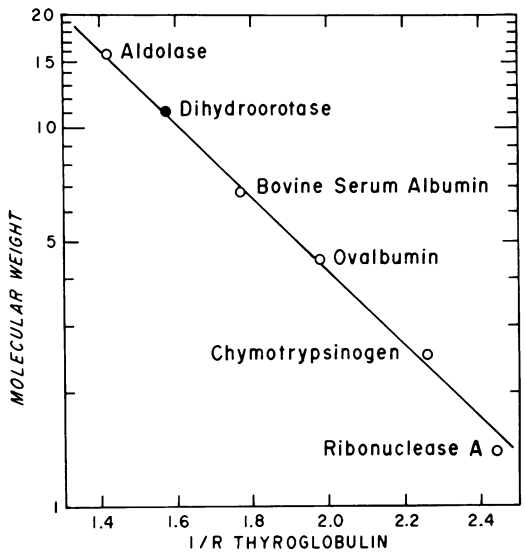

FIG. 5. *Estimation of the subunit composition of dihydroorotase using SDS-polyacrylamide gel columns. The procedure is given in the text, using the SDS gel system of Weber and Osborn (28). Mobility was determined relative to the migration of cytochrome c. Molecular weight markers: bovine serum albumin, 68,000; ovalbumin, 45,000; pepsin, 35,000; trypsin, 23,000. The molecular weights are expressed in units of 10^4 g/mol.*

TABLE 2. *Zinc content of dihydroorotase preparations from C. oroticum*[a]

Fraction	Zinc content (ng-atoms of Zn/mg of protein) of:	
	Sample 1	Sample 2
Supernatant fluid (150,000 × *g*, 90 min)	3.0	3.0
Sephadex G-25 eluate		5.5
DEAE-Sephadex eluate, 0.35 M NaCl	9.0	13.0
Hydroxyapatite eluate, pH 6.8	26.0	31.00

[a] A sample (0.1 ml) from each purification step was diluted to 1 ml and dialyzed overnight against 500 ml of buffer followed by analysis of zinc at 213.86 nm by atomic absorption spectroscopy. Sample 1 was dialyzed against 0.025 M Tris-citrate buffer (pH 7.8), and sample 2 was dialyzed against 0.025 M Tris-hydrochloride buffer (pH 7.6). Samples 1 and 2 are from different purification sequences.

of zinc to protein obtained during purification. As shown in Table 2, the ratio of zinc to protein increased approximately tenfold from the 150,000 × *g* supernatant fluid to the purified enzyme. The greater increase in specific activity (40-fold) is to be expected if other zinc-containing proteins were present in the 150,000 × *g* supernatant.

To determine the mole ratio of zinc to protein, the zinc concentration of purified dihydroorotase was determined by atomic absorp-

tion spectroscopy using three separate procedures: (i) analysis for zinc and correction for the known concentration of zinc in the storage buffer (TZ buffer), (ii) removal of unbound zinc by dialysis against 0.025 M Tris-citrate buffer (pH 7.8), and (iii) removal of unbound zinc by gel filtration on a Sephadex G-25 column. Figure 6 illustrates the protein elution pattern and zinc content of concentrated purified dihydroorotase passed through Sephadex G-25 to remove unbound zinc. The concentration ratio of protein to zinc was constant within 11% across the peak, indicating the presence of zinc in the enzyme. The three procedures used gave ratios of zinc to protein of 3.47, 3.49, and 3.83, respectively. Similar results were obtained with six preparations of dihydroorotase. Thus, the presence of four zinc atoms per molecule of native dihydroorotase is indicated. Control experiments in which bovine serum albumin was mixed with TZ buffer and subjected to procedures (ii) and (iii) showed that zinc was completely removed from this protein.

Even though preservation of enzymatic activity during purification was dependent upon the presence of added zinc, the loss of enzymatic activity was not accompanied by loss of zinc. The data in Table 3 demonstrate the loss of enzymatic activity versus time of dialysis, using buffers ranging from pH 5.8 to 8.0. The decay of activity was most rapid at pH 5.8. However, even after 10 h of dialysis, a value of

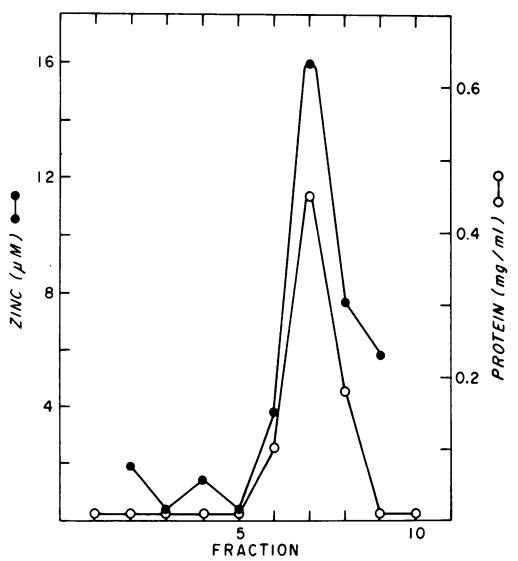

FIG. 6. *Elution of dihydroorotase protein and zinc from a column of Sephadex G-25. Zinc concentration was determined by atomic absorption spectroscopy and protein concentration by the method of Lowry* et al. *(14).*

TABLE 3. *Effect of dialysis at various pH values on dihydroorotase activity*[a]

Time of dialysis (h)	Activity (%) remaining at:			
	pH 5.8	pH 6.5	pH 7.0	pH 8.0
0	100	100	100	100
2	18	66	104	72
4	2	62	84	68
6	0	38	67	64
8	0	30	42	42
10	0	16	36	30

[a] Enzyme (specific activity, 29.18) was diluted into the appropriate buffer, and samples (1 ml) were placed in a Crowe-Englander Micro-Dialyzer. Samples (0.1 ml) were removed at the indicated times and assayed for enzymatic activity by the direct assay, as outlined in the text. For pH 8.0, 0.1 M Tris-hydrochloride buffer was used, and 0.1 M Tris-acetate buffers were used for the other pH values. All buffers contained 0.2 M NaCl.

3.4 to 3.6 g-atoms of zinc per mol of enzyme was measured at each pH value.

Treatment of 64 μg (1.5 ml) of purified dihydroorotase with 0.2 g of Chelex-100 for 30 min at pH 7.6 or 10 resulted in a 20% decrease in activity, but only unbound zinc was removed by this treatment. Further stirring of enzyme with Chelex for an additional 12 h showed negligible further loss of enzyme activity, and no removal of zinc from the protein could be found. Chelex treatment of a solution of bovine serum albumin (0.1 mg/ml) and 10 μM $ZnSO_4$ for 30 min under the same conditions removed 97% of the zinc. Removal of the Chelex from the enzyme solution resulted in complete restoration of enzymatic activity after 8 h. It appears that the decrease in activity was not due to protein-Chelex combination but may be due to reversible changes in the structure of the enzyme protein.

Since enzyme activity was readily lost at acid pH (Table 3), an experiment was performed to see whether prolonged exposure of the enzyme to pH 5.9 would release zinc. A 98% loss of dihydroorotase activity was found when 0.32 mg of purified enzyme was incubated in 5 ml of 0.025 M Tris-acetate buffer, pH 5.9, for 18 h but, even after treatment of this mixture with Chelex-100, 3.4 g-atoms of zinc per mol of enzyme was still present. Thus, zinc appears to be firmly bound to the purified enzyme, and loss of activity at acid pH is probably due to a change in amino acid charge or protein conformation near the active site. The only treatment we have found that will remove zinc from the protein is incubation of the native enzyme with SDS in the presence of mercaptoethanol. Denaturation of the enzyme by this procedure fol-

TABLE 4. *Effect of 1,10-phenanthroline and EDTA on dihydroorotase activity*

Expt conditions[a]	Inhibitor used	Inhibitor concn (mM)	Enzymatic activity[b]	Inhibition (%)
A. Incubation of enzyme with inhibitor, followed by initiation of assay with substrate	None	0	13.9	0
	OP	2	6.1	56
		8	3.3	76
	EDTA	5	9.7	30
		100	4.6	67
B. Initiation of assay by addition of enzyme preparations	None	0	15.0	0
	OP	2	13.9	7.4
		8	11.8	21.3
	EDTA	5	12.9	13.8
		100	10.2	32.0
		300	8.8	41.4

[a] Procedures used in parts A and B are listed in the text.
[b] Expressed as nanomoles of L-ureidosuccinate formed per minute per milliliter of reaction mixture.

lowed by Chelex-100 treatment results in a zinc-free protein.

Inhibition by metal chelators. Since zinc appears to be necessary for the protection of dihydroorotase activity during enzyme purification, the metal chelators OP and EDTA were tested as possible inhibitors of dihydroorotase activity. Both OP and EDTA inhibited the enzymatic conversion of L-dihydroorotate to L-ureidosuccinate, and the percentage inhibition was dependent on inhibitor concentration (Table 4). A comparison of parts A and B of Table 4 demonstrates the importance of the order of addition of substrate, enzyme, and inhibitor. For example, if enzyme is treated with 2 mM OP for 5 to 30 min prior to the addition of substrate, a 56% inhibition results; however, only a 7.4% inhibition is observed when 2 mM OP and substrate are mixed prior to the addition of the enzyme. Similar effects are apparent with EDTA. Separate experiments showed that the full amount of inhibition was achieved by less than 5 min of incubation of enzyme with inhibitor in the absence of substrate. Thus, inhibition appears to be instantaneous and the substrate protects the enzyme from the metal chelators in some manner—possibly by rapid binding of substrate to the active site and, thus, preventing the binding of the OP and EDTA.

The absorbance of OP at 230 nm prohibited its use in inhibition studies on the enzymatic conversion of L-ureidosuccinate to L-dihydroorotate. Although EDTA also absorbs at 230 nm, concentrations up to 5 mM can be used. The effect of this concentration of EDTA on the rate of L-dihydroorotate formation was measured at pH 6.0 and 7.2, with strikingly differing results: a 35% inhibition at pH 7.2 but no inhibition at pH 6.0. Thus, at least for 5 mM EDTA, the percentage of inhibition is approximately the same whether enzymatic activity is measured as L-dihydroorotate synthesis or hydrolysis.

In the direction of dihydroorotate formation, the enzymatic activity in the presence of EDTA was the same whether enzyme or substrate was used to initiate the reaction. Incubation of the enzyme with and without EDTA for 30 min at pH 6.0 reduced enzyme activity by 35%. No change in enzyme activity was observed when the enzyme was incubated for 30 min at pH 7.2 prior to the addition of ureidosuccinate, but the 35% inhibition again was obtained when EDTA was included with the enzyme under the same conditions. Although dilution effects must be taken into consideration, the loss of activity at pH 6.0 is probably due to the unstable nature of the enzyme at acid pH and not due to the effect of EDTA.

DISCUSSION

Although the involvement of a metal ion cofactor in the degradative dihydroorotase from *C. oroticum* has been suspected for some time, definitive proof has been lacking because the enzyme had not been purified to homogeneity. Sander et al. (22) repeatedly observed a decrease in specific activity during protamine sulfate and ammonium sulfate fractionation of extracts from *C. oroticum*. The activity lost could be partially restored by addition of a cofactor that was stable to acid, heat, and ultraviolet light. Enzymatic activity in these preparations was decreased further by treatment with various metal chelators, and the addition of zinc sulfate or cobalt chloride to the preparations restored the dihydroorotase activity.

The present study confirms that the stability of dihydroorotase activity during purification is dependent upon zinc. However, our work indicates that stimulation of dihydroorotase activity by zinc depends upon the method used to purify the dihydroorotase protein.

Proof that the dihydroorotase from *C. oroticum* is a zinc-containing metalloprotein is provided by our analysis of purified enzyme preparations using atomic absorption spectroscopy. When enzyme was analyzed for zinc content, we obtained an average ratio of 3.6 g-atoms of zinc per mol of dihydroorotase protein. Considering the uncertainties in the zinc and protein analyses, it seems reasonable to postulate 4 g-atoms of zinc per mol of enzyme and, therefore, 2 g-atoms of zinc per mol of subunit. This enzyme differs from most of the other zinc-containing enzymes, such as carbonic anhydrase, carboxypeptidase, and alkaline phosphatase, which have 1 g-atom of zinc per mol of subunit. It is possible that the two subunits obtained from dihydroorotase actually are dimers, which are difficult to dissociate, since it is known that SDS treatment of a protein, even in the presence of a reducing agent, may not dissociate it completely into its smallest subunit components.

Alternatively, dihydroorotase may resemble horse liver alcohol dehydrogenase, which also contains 4 g-atoms of zinc per mol of enzyme protein. Recent X-ray diffraction studies have shown that horse liver alcohol dehydrogenase is a dimer containing 2 g-atoms of zinc per mol of subunit protein (2). Studies with chelating agents and the rates of exchange between ^{65}zinc chloride and nonradioactive zinc have indicated that zinc is involved in two different roles in alcohol dehydrogenase. Two zinc atoms have been shown to be involved in catalysis, whereas the remaining two zinc atoms are thought to be involved in structural bonding of the protein subunits (7, 8). Our work gives no direct evidence what role zinc might play in dihydroorotase activity. However, removal of OP from the enzyme by dialysis relieves inhibition. Thus, it would appear that OP may be reversibly bound at the active site. Although dihydroorotase activity is readily lost when the zinc concentration is lowered or absent during fractionation procedures, zinc appears to be firmly bound to the purified enzyme and has been removed only after denaturation of the dihydroorotase protein.

Although the activity of dihydroorotase stored in phosphate buffer is stimulated by the addition of 50 μM zinc, neither the degradative dihydroorotase stored in Tris buffer, the biosynthetic enzyme from *E. coli* B (21), pea plants (15), nor rat liver (12) was stimulated by metal ions or inhibited by EDTA. The lack of EDTA inhibition with these enzymes could be due to a stronger enzyme-metal binding than the metal-EDTA complex at pH 6.0 where activity was tested. We have observed inhibition of dihydroorotase activity by both OP and EDTA at alkaline pH but not in our standard assay buffer (MES-NaOH) at pH 6.0. Sander et al. (22) reported inhibition at pH 6.0 in phosphate buffer; therefore, this discrepancy may be due to differences in the buffer used.

The purified biosynthetic dihydroorotase from *E. coli* B was purified with 10 μM zinc sulfate present in all buffers (21). When zinc

TABLE 5. *Properties of purified dihydroorotase from various sources*

Source (enzyme function)	Property						
	Molecular weight	Subunit composition	K_m values (mM)		pH optima		Metal ion (stimulation or requirement)
			N-carbamyl-L-aspartate	L-Dihydroorotate	*N*-carbamyl-L-aspartate ↓ L-dihydroorotate	L-Dihydroorotate ↓ *N*-carbamyl-L-aspartate	
C. oroticum (pyrimidine degradation)	110,000	2 Identical subunits (molecular weight, 55,000)	0.13	0.07	6.0	8.2	Zn^{2+} (or Co^{2+})
E. coli (21)[a] (pyrimidine biosynthesis)	76,000		0.76		5.0	8.5	None
Pea seedlings (15) (pyrimidine biosynthesis)	110,000		6.2	1.5	6.0	8.0	None
Rat liver (12) (pyrimidine biosynthesis [?])			0.12	0.04	5–6	9–10	None

[a] Reference numbers are in parentheses.

sulfate was present in all buffers during purification of the degradative dihydroorotase in our laboratory, we observed no zinc stimulation of the purified enzyme. Since no biosynthetic enzyme protein has been analyzed directly for zinc, the data available are insufficient to preclude the possibility that some may be metalloproteins.

There are a number of similarities between the degradative dihydroorotase from *C. oroticum* and the biosynthetic enzymes from *E. coli* B, pea plants, and rat liver (Table 5). Although the K_m values differ considerably, each dihydroorotase appears to exhibit a higher affinity for L-dihydroorotate than for L-ureidosuccinate. For a partially purified preparation of the degradative dihydroorotase from *C. oroticum*, Sander et al. (22) reported a K_m for L-ureidosuccinate of 0.8 mM, whereas we report a K_m of 0.13 mM. This discrepancy may be correlated with the fact that their purification procedure resulted in a very low recovery of dihydroorotase activity, possibly indicating partial denaturation of the enzyme and, therefore, a lowered affinity for L-ureidosuccinate. Lieberman and Kornberg (13) measured the K_m of the degradative dihydroorotase in crude cell-free extracts from cells of *C. oroticum* and obtained a value of 0.28 mM for L-ureidosuccinate, which is much closer to the value obtained in our laboratory. Another similarity between all of the dihydroorotase preparations is that for each enzyme the pH optimum is near 6.0 for the conversion of L-ureidosuccinate to L-dihydroorotate, whereas the optimum for the reverse reaction (L-dihydroorotate to L-ureidosuccinate) is in the region of pH 8.0.

Yates and Pardee (29) were the first to report that dihydroorotase activity was dependent upon the carbon and energy source used for cultivation of *C. oroticum*. When orotate was used, there was a high level of dihydroorotase activity in cell-free extracts, whereas dihydroorotase activity of extracts from glucose-grown cells was near the lower limits of detection for their assay. Thus, there may be two different enzymes catalyzing the same reaction, as we have demonstrated for another enzyme (dihydroorotate dehydrogenase) of the pyrimidine pathway (24-26). Alternatively, it is also possible that growth on orotate causes an increased production of the dihydroorotase protein normally involved in pyrimidine biosynthesis. Such a finding has been reported for the arginine pathway in *Pseudomonas aeruginosa* (27). Growth of this organism on arginine-containing medium induces the synthesis of high levels of an enzyme normally involved in arginine biosynthesis.

We have observed that the dihydroorotase (molecular weight, 110,000) and dihydroorotate dehydrogenase (molecular weight, 115,000) proteins move with almost the same rate in polyacrylamide gels. Since the molecular weights are nearly identical and migration rates in polyacrylamide gels indicate the net charge must be almost the same, the amino acid composition of the two proteins may be quite similar. Further studies on these enzymes and their subunits may reveal close relationships in primary structure. These studies may help us to understand the evolution of the pyrimidine pathway and to explain some features of its regulation.

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